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THE
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THE ACTION OF SAPONIN AND ITS NON-HEMOLYTIC MODIFICATION ON GROWTH[1]

JOSEPH M. BUTROS

Biology Department, American University of Beirut, Beirut, Lebanese Republic

The hemolytic activity of saponins has been extensively studied and has stimulated investigations related to other properties of these compounds. A series of studies on seed germination and plant growth are reported by Balansard and Pellissier (1943; 1944; 1945) and indicate a growth-promoting activity of saponin in very dilute solutions (10 to 100 ppm.). Such solutions produce considerable proliferation forming tumor-like concrescences in ivy shoots (1943). Seedlings from treated cereal seeds grow faster and become larger than their controls (1944). Similarly the germination of tomato seeds was accelerated (1945). Dilute saponins accelerate water absorption of moistened seeds and speed up germination of corn (Balansard, Pellissier and Conil, 1946).

On the other hand, the inhibitory action of saponins in higher concentration has been reported by some authors. Amerio and Dalla (1942) found that the roots of *Allium cepa* and coleoptiles of oat were irreversibly but non-specifically damaged. Von Euler (1946) found that seedlings of *Lepidium sativum* and *Hordeum vulgare* grew to only one-third to one-tenth of the lengths of the controls. Inhibitory action is also reported in animals: heart and lung tissue of hen embryo (Hideo, 1928) and Jensen sarcoma (Frey, 1938).

Two years ago the author described the cytolytic effects of 0.5 per cent Merck saponin on rat tissue and tumors (Butros, 1948). The Feulgen stain showed degenerative changes in the nucleus. This suggested a nucleo-toxic action and the necessity of a study of the effects of lower concentrations, especially on growing tissues. As onions have often been used in the study of mitosis and growth, they were here also employed in the greater part of these experiments.

Another aspect of saponin action was investigated also, *i.e.*, the possibility that its nucleo-toxic action is independent and separate from its hemolytic action. Hemolysis can be inhibited by a combination of saponin with cholesterol (as will be discussed further on). The nuclear damage might be another matter, and might depend on the action of saponin on some constituent other than those involved in hemolysis. If so, its action might show itself by interfering with mitosis and growth even if it has lost its hemolytic power. After sufficient indications of inhibitory action of both saponin and its "cholesteride" were evident by trials with onion roots,

[1] Supported by a grant from the Research Committee of the School of Arts and Sciences of the University.

the author started investigations on animals. The toxicity of the "saponin cholesteride" was much less than saponin of the same concentration and so it was felt safe enough to test it on tadpole development. Experiments with a non-hemolytic saponin-eosin preparation are also described.

The Saponins Used

Two saponins have been used in this work: Merck's (white pure), and Baker's (yellow). Solutions were made in tap water for the onion roots, in distilled water for the others.

The hemolytic strength of these two saponins was not the same. Hemolytic tests in this paper refer to the standard method of Ponder (1948). The time of complete hemolysis is the one considered except in some cases, especially of "saponin cholesteride," where an approximation of the percentage of hemolyzed r.b.c. by the microscope was employed.

Baker saponin in 1/1000 dilution resulted in complete hemolysis in three minutes. A 1/10,000 solution needed 18 hours to complete hemolysis, but a solution of 1/15,000 brought about only 75 per cent hemolysis in the same length of time. Merck saponin solutions of 1/50,000 show complete hemolysis in 18 hours; 1/100,000 show 50 per cent hemolysis in 18 hours and 1/200,000 show 25 per cent hemolysis in the same time.

Action of Saponin on Onion Root Growth

Onions (*Allium cepa*) were sprouted in water before placing them in the saponin solutions. Whole and half onions were used, the sister halves of the treated being used as controls in water. Measurements of root lengths were made and sample roots were fixed for cytologic preparations. Roots that had stopped growth in saponin were returned to water to determine their ability to resume growth. Very dilute solutions of saponins were included in this study to compare with the work of Balansard and Pellissier referred to in the introduction.

Inhibitory Concentrations

An arrest of root lengthening was definitely established within one or two days, depending on individual susceptibility, after placement in 0.05 per cent to 0.1 per cent Merck saponin and 0.25 per cent to 0.3 per cent Baker saponin. If roots were returned to water at this point, most of them were able to resume growth, but if left more than four days in the saponins they could not. The new growth was easily distinguished by its fresh, white texture as compared with the brownish treated tips. There was often a knot-like hypertrophy at the junction between the area grown in saponin and that grown in water. Curvature of the roots was noted in saponin but not in the controls. If left in the solutions over a week, the arrested roots developed secondary roots that came to a standstill in a couple of days. New roots from the periphery of the onion sometimes developed (Fig. 1).

From the study of sections taken at intervals of 12 hours for five days in saponin, it became clear that the mitotic process was inhibited completely by the second day, when no mitotic process was found. All cells were in the resting stage or in what appeared as very early prophase (Fig. 2). The chromatin particles were small,

thin rods. No disturbances or aberrations were noticed in the mitotic figures of the first day except that they were somewhat less numerous than the control. The region of elongation was not modified. Comparisons between microscopic and length studies were in agreement; roots that resumed lengthening after return to water showed recurrence of normal mitoses as abundant as the controls.

FIGURE 1. Onion that was replaced in water after treatment with 0.1 per cent Merck saponin, showing arrested roots with knotty beaded tips and secondary growth.

Lethal Concentrations

If roots were left long enough (4 to 5 days) in an inhibitory concentration, or for two days in a higher concentration, cytolytic degeneration occurred, ending in death of the desiccated roots. The nuclei became first pycnotic, then shrunken, pointed and eccentric. The cytoplasm gradually vacuolated out. Cells at the root cap were also displaced and disintegrated.

Sub-Inhibitory Concentrations

Experiments were done as described above: half onions were placed in the saponin solutions and their control halves in water. The length that represented

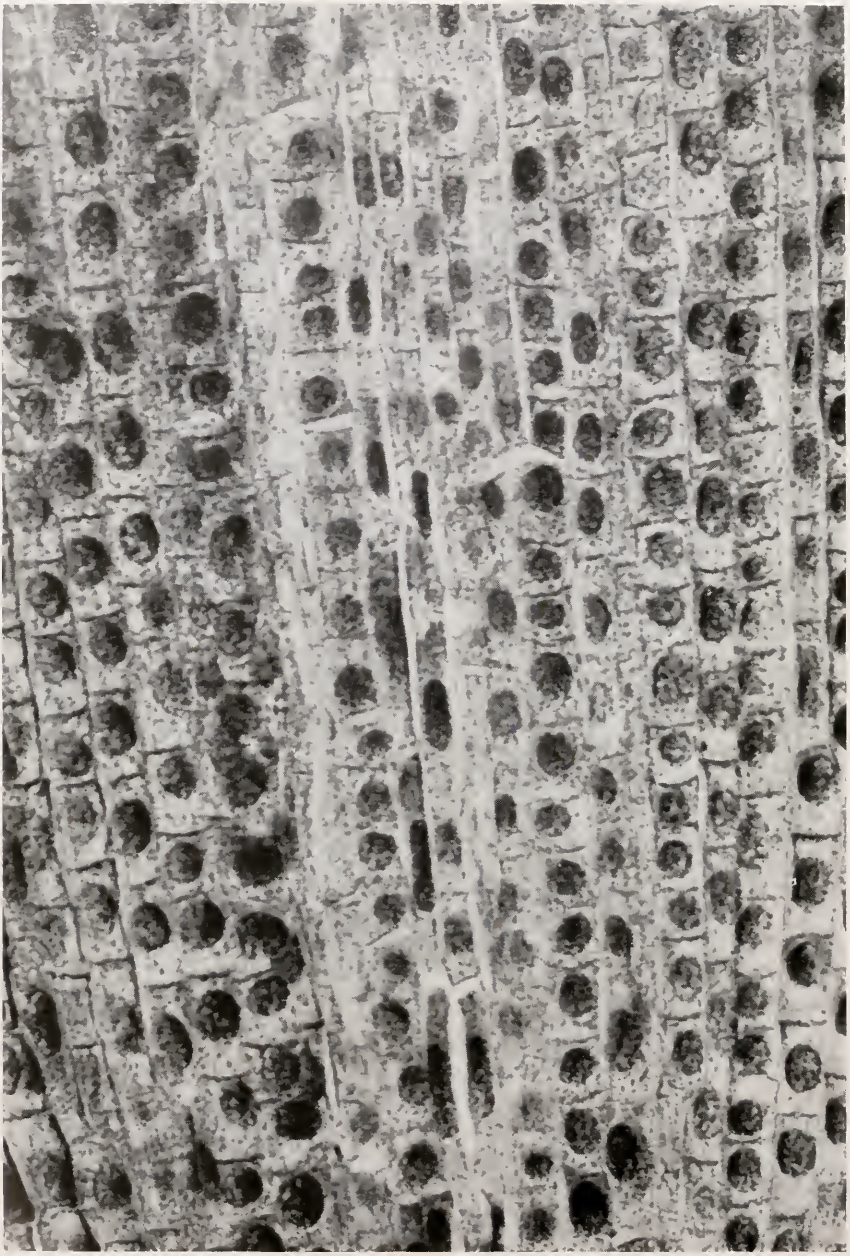

FIGURE 2. Section of onion root showing inhibition of mitosis in 0.1 per cent Merck saponin; fixed in Bouin's and stained with hematoxylin and eosin. × 400.

the majority of the roots in each specimen was tabulated daily, from which increments were calculated. The concentrations used were:

Merck: 1/5000; 1/10,000; 1/100,000; 1/500,000
Baker: 1/1000; 1/2000; 1/10,000; 1/500,000; 1/1,000,000; 1/2,000,000

As a summary, Table I gives the length measurements and compares the growth rates of the various concentrations of Baker saponin.

For a check on the deviation between normal onion halves an experiment was run with onion halves in two sets of vessels, both containing water. The average difference in length between the two sets at the end of 12 days was 0.34 cm. This value was used in evaluating the significance of experimental results.

TABLE I

Growth of onion roots in various concentrations of Baker saponin

No. of Days in Solution	Concentration	Length in Saponin	Length in Water	Increase in Saponin	Increase in Water	Growth Index*
7	1/2,000,000	9.9 cm.	8.9 cm.	8.9 cm.	7.9 cm.	1.1
10	1/500,000	11 cm.	9.3 cm.	10 cm.	8.3 cm.	1.2
6	1/10,000	8.2 cm.	9.4 cm.	7.2 cm.	8.4 cm.	0.9
8	1/2000	7.1 cm.	9.4 cm.	6.1 cm.	8.4 cm.	0.71
5	1/1000	5.1 cm.	8.4 cm.	4.1 cm.	7.4 cm.	0.53

* Increment per day in saponin divided by increment per day in water.

On examining Table I it will be seen that the very dilute concentrations of Baker saponin may be considered to favor growth. This agrees with the findings of Balansard and Pellissier referred to above. The lowest dilutions of Merck saponin used in these experiments did not show this growth-promoting power. As Merck saponin is stronger, they really compare to higher concentrations in terms of Baker saponin, which are also non-growth promoting.

Microscopic examination of root sections treated with the sub-inhibitory solutions of saponin showed apparently normal mitotic figures and healthy cells. The sub-inhibitory dose, however, is capable of producing inhibition of mitosis and cytolysis of cells if the roots were left long enough in the solution. After 6 days in 1/10,000 Merck saponin, the cells were vacuolated and no mitotic figures were discernible.

The Respiration of Onion Roots in Saponin

Trials were made to find out if saponin suppressed the respiration of onion roots. If it did, its inhibitory action could have been due to, or at least related to such action. The indicator bromthymol blue was used in most cases. This was very reliable as shown by repeated trials with growing roots, roots killed in alcohol, and roots immersed in KCN. It was found that in 0.3 per cent Baker saponin (which is sufficient to stop mitosis in two days), the roots were able to decolorize bromthymol blue, indicating production of an acid; pH measurements of the solutions containing the onions served as a further check on the production of carbon dioxide. The pH of the saponin solution in which onions grew was 6.4. Its control with no onion was

7.4, and a second control with a dead onion was 7.6. Further, distilled water containing respiring onions had a pH of 5.4 on the second day; its control with no onion was 6.0. These results show that onion roots continue to respire in saponin while their mitotic process is gradually coming to a standstill.

Preparation of Non-Hemolytic "Saponin Cholesterides"

In the extensive article on saponins by Kobert-Rostock (1924), it is explained that all saponins lose their hemolytic activity when their solutions are warmed with cholesterol. Windaus (1909) made a study of this reaction by using a steroid saponin, digitonin, which, although different in structure from the ordinary saponins, shares in their hemolytic activity. He made a digitonin cholesteride compound by precipitating it from an alcoholic solution of the two compounds. The cholesteride is not separated into its constituents by ether but is separated by boiling xylene. Using ordinary saponin, Meyerstein (1910) rendered it non-hemolytic by shaking it with cholesterol in normal saline. He stated that it is not of great importance whether these two compounds were held up by a genuine chemical combination or only by adsorption. His preparations were freshly made when needed.

The author used a modification of Meyerstein's method, which gave a stable non-hemolytic suspension that could stand over two months without splitting back into saponin and cholesterol. The method depended upon the use of a homogenizer [2] which ground the cholesterol into very fine particles. This was run into hot saponin solution and the reaction (or adsorption) took place instantaneously, as shown by the loss of frothing and loss of hemolytic action. Comparison with hemolytic tests made on saponin was very helpful in determining approximately the amount, if any, of free saponin in the suspension, and so indicating the possibility that the biological action might be due to free saponin.

A phosphate buffer was sometimes added to the "saponin cholesteride" when it was used with tadpoles. This seemed to favor the adsorption of the two components and make the compound more stable. The proportions of the components used were:

Baker saponin 3 gm. per liter; cholesterol 1.5 gm. per liter
Merck saponin 3 gm. per liter; cholesterol 2.5 gm. per liter

Saponin did not become non-hemolytic except when there was an excess of cholesterol. The suspension remained milky and non-hemolytic for over two months.

Properties of the "Saponin Cholesteride"

If the suspension is prepared successfully, it does not froth, is milky with the larger particles settling down, but it does not clarify even within three months. Merck's preparation is white, and Baker's light brown. Sometimes, for unknown reasons, it was not possible to make the suspension as there was a flocculation. The clear fluid, however, of the flocculated preparation was non-hemolytic and had the same inhibitory property as the milky suspension.

The clear filtrate of the "saponin cholesteride" remained non-hemolytic for a few days during which it was inhibitory to onion roots like the mother compound.

[2] The author is deeply indebted to Messrs. Awad and Baramki of the Chemistry Department for their help in this matter.

The color of the filtrate of the Baker preparation was pinkish, whereas the color of solutions of this saponin was yellow and never pink, no matter how dilute. The clear filtrate does not froth also, another indication that it does not contain free saponin. It could have been a saponin-cholesterol complex.

Extraction with ether of the Merck cholesteride until all excess cholesterol was removed gave a milky suspension with no microscopically visible crystals of cholesterol. This extract was non-hemolytic also (after evaporation of the hemolytic ether), but it became hemolytic on standing a few days, possibly due to the lack of excess cholesterol.

Thus it seems that the "saponin cholesteride" prepared in this manner was of variable and uncertain composition, and deserves a detailed study.

Action of "Saponin Cholesteride" on Onion Roots

Whole and half onions were placed in the suspension; controls were in water and in suspended cholesterol. In all cases, concentrations refer to the amount of saponin in the suspension.

Results in Baker Saponin

A 1.8 per cent suspension checks the growth in length of the roots in two days. The tips have the knotty appearance which was noted in saponin, and they curve upwards. A 0.9 per cent suspension was also able to check the growth of the roots within two days. Microscopic examination revealed the absence of mitotic figures, all cells being in the resting stage. Signs of beginning degeneration were apparent on the third day, indicated by a very little vacuolation of the cytoplasm and slight shrinkage of nuclei. A few roots with more resistance showed four or five mitotic figures per field ($\times$ 250). In the 0.3 per cent suspension, mitotic figures were found in the slides but about 10 per cent less than in the control.

Results with 0.3 Per Cent Merck "Saponin Cholesteride"

This concentration was insufficient to stop growth and mitosis completely. However, there was a 50 per cent reduction in the number of mitoses as compared with water control, and with dilute saponin control. The rate of lengthening dropped to 30 per cent. The roots were thicker than usual and the microscopic view indicated hypertrophy of the cells. It can be assumed from trials with Baker saponin that if the concentration were raised, complete arrest would result.

Proof That the Action is Not Due to Free Saponin

The hemolytic power of various concentrations of the "saponin cholesteride" was determined to help in approximating the amount, if any, of free saponin in the cholesteride. It should be remembered that complete hemolysis occurs within 18 hours in a concentration of 1/10,000 of Baker saponin but not if the concentration is 1/15,000 or 1 20,000. As the concentration of saponin required to stop mitosis is 0.3 per cent, which causes hemolysis in a few minutes, and as the "saponin cholesteride" did not show complete hemolysis in a much longer period (24 hours), there is no possibility of its action being due to saponin. Similarly, in the case of Merck saponin, there is immediate complete hemolysis in up to one per cent. The mitotic inhibitory dose is within this range, and as there was no complete hemolysis of the

"cholesteride" overnight, it is out of the question that the inhibition (partial in this case) was due to free saponin.

Daily hemolytic tests were made from samples of "saponin cholesteride" in which onions were growing, to eliminate the possibility that any saponin was splitting from the cholesteride as a result of the action of some agent from the onion. No splitting took place when only the roots were immersed in the suspension.

Filtered Merck "saponin cholesteride" (0.3 per cent) did not cause complete hemolysis in 24 hours. It arrested the growth of onion roots after a growth of ½ cm. during the first day.

Controls

Onions were grown in cholesterol suspension made by the homogenizer of the same strength as the 0.3 per cent cholesteride (which is partially inhibitory). The rate of growth in length was as follows:

In water	1 cm. per day
In cholesterol	0.8 cm. per day
In "saponin cholesteride"	0.4 cm. per day

In another experiment, corresponding half onions were placed in cholesterol and water respectively. The cholesterol in this case was ground in a mortar and then shaken several hours by a shaking machine to the same strength as the "saponin cholesteride." There was no apparent difference between the length of roots in cholesterol with those of the water.

Curvature of Onion Roots

Root curvature in saponin and its "cholesteride" suggested interference with auxins, although the latter are concerned with root sprouting rather than with root growth (Went and Thimann, 1937). If the "cholesteride" interferes with auxin action, then it might prevent the sprouting of roots, so onions were placed in these solutions. In both saponin and the "cholesteride," the onion roots appeared and grew for two to three days. However, they stopped at the length of 1.5 cm., as they usually do if placed after sprouting. It does not seem that curvature in these solutions had a relation to auxin action.

Toxicity of "Saponin Cholesteride" to Animals

A comparison of the toxicity of saponin and its "cholesteride" of the same concentration was made by noting their killing time on certain animals. In general the saponin toxicity was greatly decreased. For example, paramecium is cytolyzed instantaneously in 0.45 per cent Baker saponin, whereas it continued to move about for 5 hours in double that concentration of "cholesteride."

Table II compares the duration of life in Baker saponin and its "cholesteride" for the fresh water snail *Melanopsis bovieri* and planaria.

Toxicity studies were also made on tadpoles, and it was clear that the toxicity was greatly reduced when compared to that of pure saponin. It took 12 hours to kill tadpoles in 0.9 per cent "saponin cholesteride," whereas saponin of that concentration killed them in a few minutes. The "cholesteride" was non-hemolytic and this property was tested daily and in particular during the interval that the tadpoles were

in the suspension. This is significant as it points to a toxic property independent and separate from the known hemolytic property.

Filtered Merck "cholesteride" (0.2 per cent) kills tadpoles in 5 hours and shows complete hemolysis in less than 20 hours at 37° C. At room temperature it does not show complete hemolysis in that time, however. The ether-extracted

TABLE II

Duration of life in Baker saponin of Planaria and snails

Concentration	Saponin		Saponin Cholesteride	
	Planaria	Snail	Planaria	Snail
0.9%	5 minutes	3.5 minutes	3–5 hours	1 hour
0.45%	4 minutes	4 minutes	10 hours	8 hours
0.09%	30 minutes	5 minutes	24 hours	20 hours
0.05%	60 minutes	6 minutes	40 hours	30 hours

"cholesteride," which is free of cholesterol particles, also kills the tadpoles in 5 hours and produces 50 per cent hemolysis in 20 hours. If we assume a very low content of free saponin, such as 1/200,000, which has the same hemolytic power as the above suspensions, we still cannot explain the killing action of these suspensions, because the tadpoles can tolerate this much saponin overnight.

Cholesterol Control

A suspension of cholesterol in water kills the tadpoles within 5 hours, possibly by the accumulation of crystals in their gills. If the suspension is filtered, it becomes harmless.

Cytolysis of Mammalian Tissues

Slices of liver, kidney and intestine of the rat were placed aseptically in 0.3 per cent Merck "cholesteride" and similarly in 0.3 per cent saponin for 7 hours in the ice box. Microscopic study of prepared sections revealed almost equivalent degree of cytolysis in both solutions. The liver was more damaged by saponin than by its "cholesteride," as judged by the dissolution of chromatin resulting in a cloudy homogeneous nucleus. The shrinkage of the liver tissue was enormous in both cases; many wide, long spaces were observed among the liver cords.

The intestinal epithelium was greatly dehydrated and damaged. The cells were almost devoid of cytoplasm. The nuclei were shriveled points. The effect of the "cholesteride" in this case was more striking than that of saponin.

The kidney nuclei were pycnotic in both solutions. The resistance of this organ was greater than that of the liver and the intestine.

Hemolytic tests were performed at the beginning and at the end of the immersion period of the tissue slices, and the "saponin cholesteride" was found nonhemolytic in 20 hours. It seems, then, that "saponin cholesteride" is as effective as saponin in causing the cytolysis of tissue cells.

Action of "Saponin Cholesteride" on Tadpole Development

It was mentioned under the properties of this compound that its toxicity as compared to saponin was greatly decreased, to such an extent that tadpoles could stand 0.9 per cent Baker "saponin cholesteride" for several hours with no serious damage. The tadpoles became quite irritable at first, moving rapidly in all directions, then they quieted down as if anaesthetized and remained so to the end of the period of immersion (1 to 2 hours). During this treatment their heart rate drops down to 30 beats a minute, as compared to 100 beats of the normal (chloretone anaesthetized) tadpoles. When washed and returned to water they remain "intoxicated" for an hour or so before they regain their normal activities and normal heart rate.

Tadpoles of various stages as well as cleaving eggs were treated by short immersions in "saponin cholesteride" and the effect on development was noted by comparing with the controls. All tadpoles were kept in finger bowls and fed boiled spinach leaves.

Trials with Cleavage Stages

Female frogs were induced to ovulate by injecting several pituitary glands in the abdomen of each. They were stripped into a suspension of minced testes. When all ova were in the two-cell stage, they were divided into four groups and placed in the following solutions:

1. 0.15 per cent Baker saponin
2. 0.9 per cent Baker "saponin cholesteride"
3. 0.0025 per cent KCN control
4. Water control

Within 5 hours, eggs in the four groups cleaved to the 16-cell condition. By 20 hours the eggs in the three agents reached the blastula or Stage 8,[3] while those in water reached late blastula or Stage 9. After 64 hours, both saponin and "saponin cholesteride" were still in blastula, and did not resume development after return to water. KCN treated eggs were in Stage 12 and water group in Stage 14. It was apparent that there was a generalized poisoning, due to continuous immersion in the solution, in the saponin and its "cholesteride."

Unhatched Batch (Stage 17)

These were immersed 2 hours daily in 0.9 per cent suspension for a period of two weeks. On the second day after treatment, 11 tadpoles hatched from the water control but none from the "cholesteride" group. On the ninth day, inspection of the organisms showed that the treated groups were smaller. They still had their suckers, whereas the controls had lost them. Treatment stopped after two weeks. On the fiftieth day from the beginning of the experiment, the treated animals averaged 2.8 cm. in length; their hind limbs were in Stage 7. The controls were 3.5 cm. and their limbs in Stage 11 (Fig. 3).

Hatched Tadpoles (Stage 19)

On the fifth day, it was noticed that the water control group were ahead of the "cholesteride" group in having their mouths open and their eyes more pigmented.

[3] In this paper, stage numbers are according to Rugh, 1948.

On the twenty-sixth day the tadpoles in water averaged 2 × 0.5 cm., while those in "cholesteride" were 1.4 × 0.3 cm.

Tadpoles of Stage 21

Daily immersion in 0.9 per cent "saponin cholesteride" for 15 days, after which they were returned to water. On the fiftieth day, the water controls had complete

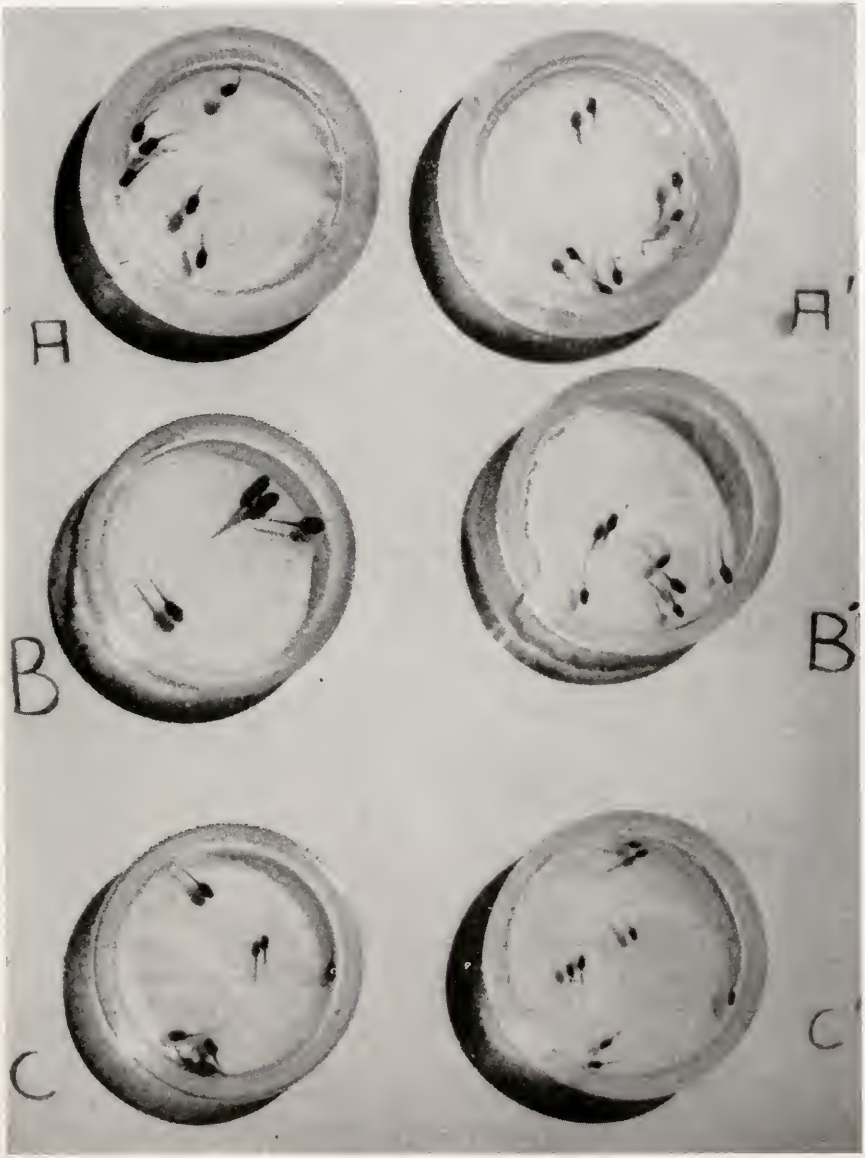

FIGURE 3. A', B', C' tadpoles showing retardation of growth by immersion in 0.9 per cent Baker "saponin cholesteride" for an average of one hour daily. A, B, C their untreated controls.

hind limbs, and beginnings of the fore limbs (Stage 19). The treated ones were around Stage 10. Among the controls there were some 4.5 cm. long and some 3.8 cm.; the treated were between 3.3 and 2.8 cm. (Fig. 3).

Tadpoles of Stage 23

In this series, the treated groups were immersed for 1.5 hours each in the same "cholesteride" as the previous cases, also for 15 days. On the eighth day, the controls had well formed jaws and four rows of teeth; the pigment covered only half the abdomen. The treated ones had one row of teeth, and much less abdominal pigmentation. Thin pieces of opercular fold were still visible. On the twentieth day the controls averaged 1.8 cm. × 0.45 against 1.2 × 0.35 cm. of the treated group (Fig. 3).

Summary

It is seen that there is a retardation in development of tadpoles due to "saponin cholesteride." Size as well as stage differences were noted. Microscopic examination of serial sections did not reveal any histologic difference in the treated animals. They were only smaller and retarded in the rate of development.

Action of Non-Hemolytic Saponin-Eosin

According to Noguchi (1906), eosin abolishes, at least partially, the hemolytic power of saponin if the mixture is exposed to the sun. The author kept a 0.3 per cent Merck saponin and 1.5 per cent eosin mixture in the sun for 65 hours. The excess eosin was adsorbed out by charcoal. Trials with this almost cleared solution showed that it was non-hemolytic and capable of retarding growth of onion roots as much as 75 per cent of the water control. Onion roots in similarly treated eosin control were retarded by 25 per cent only.

Discussion and Conclusion

The number of chemicals that can interrupt mitosis is enormous. Yet not all toxic substances do that. For example, the author tried several known poisons and found the following facts: one per cent Lysol kills onion roots but does not prevent mitoses completely. Bromine water diluted 9 times causes cytolysis and disruption of cells too drastic to show mitoses. One per cent pyridine was interesting: while cytoplasm, nuclei and nucleoli of the treated roots were in as perfect form as those of the best control roots, there were no mitotic figures at all. The roots, however, were dead. It warrants further study. Three-tenths of one per cent bile salt mixture kills and softens the roots in two days, yet there were some mitotic figures. It can be said that saponin is more than a general toxic substance, having a specific inhibition on mitosis in addition to its retardation of growth in a general way. Its non-hemolytic "cholesteride" does the same also but in higher concentrations. It is possible that this inhibitory action is the result of disturbances in the water content of the cells. There are several indications in support of this view: the shrinking and curvature of roots, actually becoming brittle, in the saponin and "cholesteride" solutions; the very hygroscopic nature of the compound; and the extensive shrinkage in animal tissues placed in saponin. The role of water in the

growth process is fundamental and a disturbance in water content might be the explanation of retardation in tadpole development and onion root growth. On the other hand, the compound might have an affinity to nuclear constituents. In fact the cytolysis of tissues in saponin, referred to previously (Butros, 1948), indicated a gradual disintegration of the nucleus as revealed by the Feulgen reaction. It is significant to note here that the "saponin cholesteride" had the same cytolytic power on mammalian tissues as saponin.

To conclude, it seems that the whole problem is worth further investigation. As most of this work was done on onion roots, more work should be turned to animal and tissue culture fields. Various other saponins might be tried and might give a milder toxic cholesteride with more inhibitory powers.

Summary

1. Onion root elongation was arrested within two days by 0.1 per cent Merck saponin and 0.3 per cent Baker saponin.

2. No mitotic figures were discerned in these cells; the nuclei of all cells were in the resting stage.

3. Lower concentrations, down to a certain point, reduced the rate of growth of the roots. Below that the very dilute saponins appeared to be growth stimulating.

4. A method of preparing a non-hemolytic suspension of saponin cholesteride is given.

5. The properties of non-hemolytic saponin cholesteride are described. Its toxicity to animals is much less than saponin; also it arrests onion root growth. It also retards the development of tadpoles. Its cytolytic power to mammalian tissues does not seem to have changed.

6. A non-hemolytic preparation of saponin-eosin is found to be partially inhibiting to onion root growth.

7. The possible explanations for this action are discussed.

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STROKE AMPLITUDE AS A FUNCTION OF AIR DENSITY IN THE FLIGHT OF DROSOPHILA

LEIGH E. CHADWICK

Medical Division, Army Chemical Corps, Army Chemical Center, Maryland

With the accumulation of the necessary quantitative information, it is gradually becoming apparent that the complex wing movement of insects may represent the integration of several components, so interrelated that variation in any one is compensated precisely by appropriate changes in the others. Thus, for example, strong evidence in support of the theory that wingbeat frequency in the higher Diptera is regulated by the loading of the flight muscles has been put forward by Pringle (1949); in the case of Drosophila the relationship between frequency and atmospheric density agrees qualitatively with this point of view, emphasizing the fact that a major element of the load is the air resistance which the wings must overcome (Chadwick and Williams, 1949). Here, however, the observed changes in frequency were insufficient to compensate completely for the variation in load imposed by density change, indicating clearly that elements of the wing motion other than the frequency must also vary in an orderly manner if equilibrium is to be preserved.

From studies with vertebrate muscle (see Fenn, 1945), as well as from the few pertinent investigations with insects (Solf, 1931; Kraemer, 1932; Cremer, 1934), it is well known that not only the speed but also the extent of muscular shortening may be reduced by an increase in load. Regular changes in amplitude resulting from alteration in atmospheric density are therefore wholly to be expected. The problem in the present study has been the investigation of such changes from the viewpoint of their contribution to the integration of the flight mechanism as a whole.

One may reason, *a priori*, that in the analysis of insect flight the stroke amplitude should be as important as the frequency with which the wings are moved, since in theory the work done per beat is proportional approximately to the square of each of these factors. Evidence supporting this deduction has been produced, as far as frequency is concerned, by measuring respiration and rate of wingbeat concurrently during flights in which the level of activity was altered progressively by fatigue (Chadwick and Gilmour, 1940; Chadwick, 1947). However, such data suggest also that, unlike frequency, amplitude does not vary systematically under these experimental conditions, an inference which Williams (1941) has confirmed photographically.

It is well known, however, that in other circumstances amplitude may change considerably. Differences in the amplitude of right and left wings were observed by Voss (1914) and Stellwaag (1916), who discovered in this asymmetry the mechanism for steering in the horizontal plane. Moreover, Hollick (1940) has measured changes in the torque about the transverse axis which were dependent upon variation in the amplitude of both wings simultaneously, so that steering in the vertical plane also appears to be achieved through control of amplitude.

That variations in amplitude are not related solely to steering is attested by various other observations. Thus, Hollick (1940), like Magnan (1934), has described an inverse relationship between amplitude and the airspeed of the insect; while Sotavalta (1947) noted that smaller amplitudes are characteristic of insects flying freely in nature than of the same species when fastened, and that amplitude often increases when an insect carries a heavier load, as may be observed in predatory species with prey or in mosquitoes after a blood meal. Some of these facts may seem at first in contradiction with the decrease of amplitude noticed in fastened Drosophila at air densities above normal (Chadwick and Williams, 1949), but probably there is no real disagreement. If one makes the likely assumption that a fastened insect is already bearing a full load, any further burden on the muscles, as from an increase in the resistance of the air to the passage of the wings, can only result in a decrease in wingbeat frequency, amplitude, or both. A free-flying insect, on the other hand, ordinarily has some fraction of power in reserve. That the stroke amplitude of fastened Drosophila does in fact change systematically with alterations in air density is demonstrated by the observations described below.

Method

The experimental method was essentially that used previously by Hollick (1940) and Williams (1941), in which the envelope of the wing motion is recorded photographically by making a time exposure of a fastened insect in flight.

Females of *D. virilis* Sturtevant were anesthetized with carbon dioxide gas, and each specimen fastened to a wire by means of paraffin. The mount was then adjusted in a cylindrical holder which carried a retractable spring platform with which the feet of the fly were placed in contact, and the holder was slipped into the flight chamber, as shown in Figure 1. Care was taken to orient the insect so that flight was in a horizontal plane, with the specimen either head-on or sideways to the camera. Flight was induced by bringing a small permanent magnet, outside the chamber, into position beneath the platform.

The chamber consisted of a glass cylinder of heavy combustion tubing, 6 inches long and one inch in internal diameter, enclosed in a frame cut from stainless steel pipe. Threaded steel fittings provided for closure at both ends. As illustrated in Figure 1, one fitting carried inlet and outlet tubes for exchanging gas within the chamber; the other served to clamp a lucite flat, 0.25 inch thick, against the end which faced the camera.

Illumination was supplied by microscope lamps, one at either side of the chamber; these were turned on only while a record was being made. Exposure times of 1 to 2 seconds, aperture of f/11, gave head-on photographs at about 1 diameter on Super-XX film from which measurements of vertical amplitude could be made satisfactorily (Fig. 2). With the insect in side view, better pictures were obtained with an exposure of 0.2–0.5 second at aperture f/16 (Fig. 3).

Pressure within the chamber was varied by adding oxygen from a commercial cylinder, or by means of a vacuum pump. Positive pressures were read from a gauge, negative pressures from a mercury manometer against which the gauge had been calibrated up to a pressure of 3 atmospheres. Higher pressures were measured by gauge alone, assuming a linear extrapolation of the calibration data. The

atmosphere within the chamber was kept moist by evaporation from a piece of dampened filter paper. After each change of pressure a few minutes were allowed for equalization of the chamber with the laboratory temperature of 27 ± 1 degrees C.

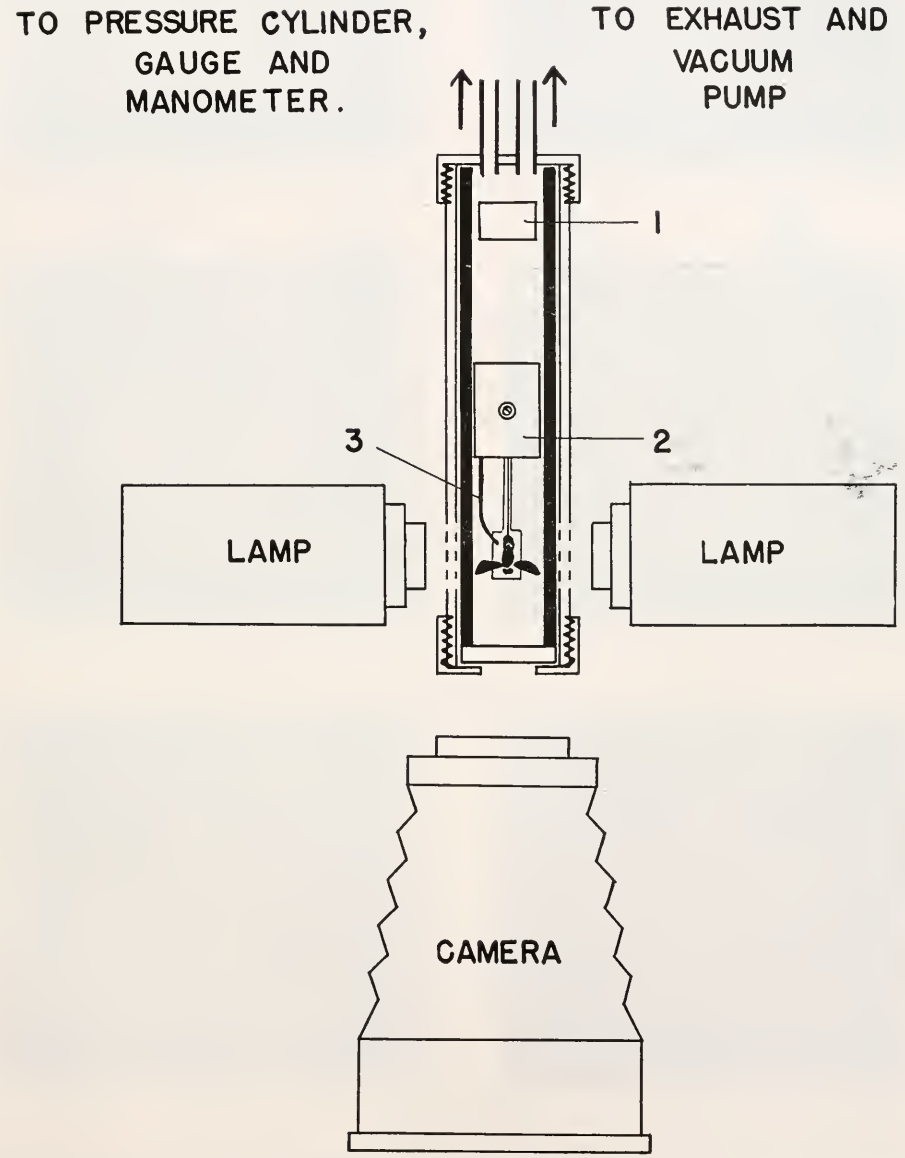

FIGURE 1. Diagram of apparatus. 1, piece of moistened filter paper; 2, holder for insect mounting and retractable platform; 3, stop for platform. For a description of other details, see text.

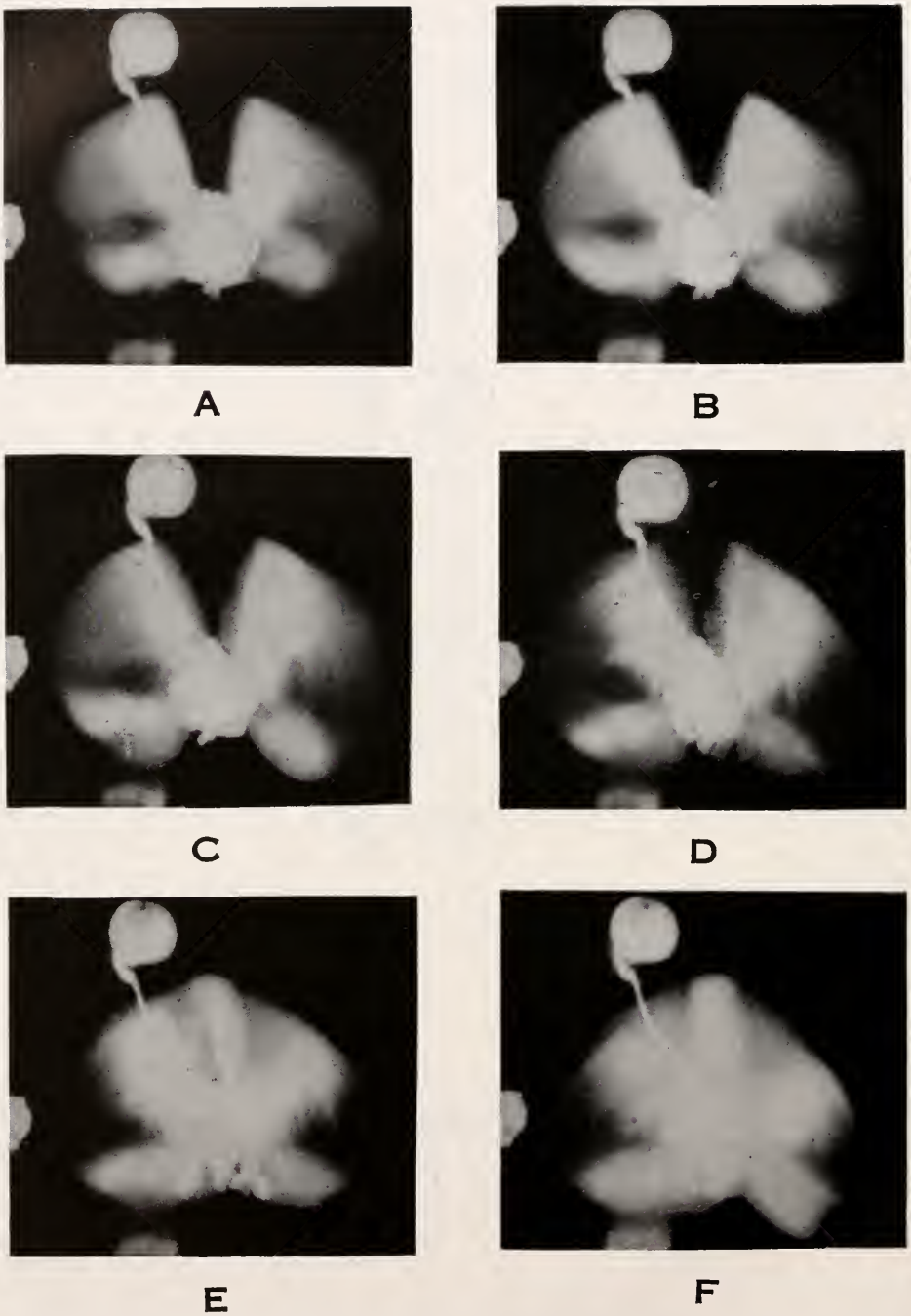

FIGURE 2. Representative series of head-on photographs to show alteration in stroke amplitude of a specimen of *D. virilis* in successive flights at different air pressures, as follows: a. 3863 mm. Hg; b. 2311 mm. Hg; c. 1520 mm. Hg; d. 760 mm. Hg; e. 380 mm. Hg; f. 190 mm. Hg. Records obtained at 3087 mm. Hg and at 95 mm. Hg have been omitted.

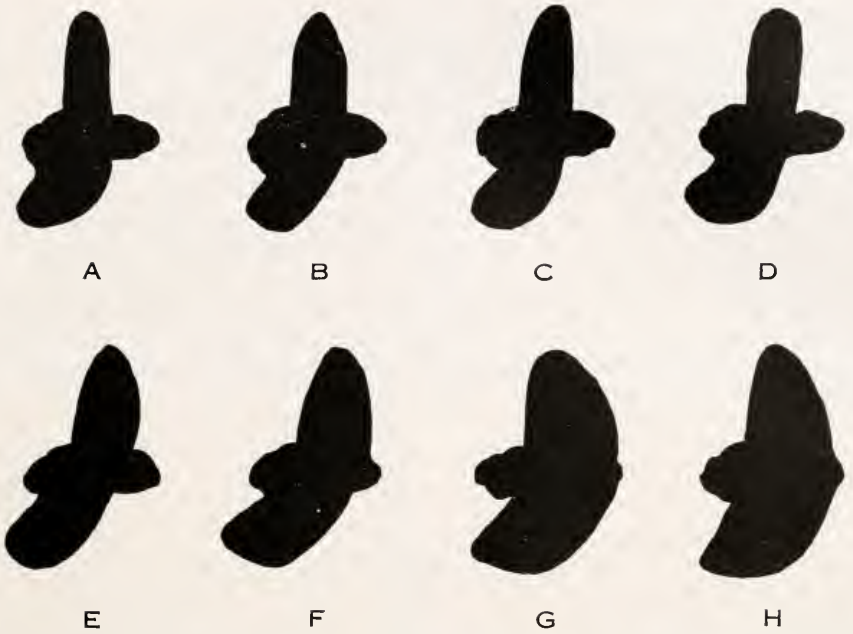

FIGURE 3. Series of silhouettes, traced from enlargements of original photographic records, to show changes in envelope of wing movement of a specimen of *D. virilis* during successive flights at different air pressures. Left lateral view. a. 3863 mm. Hg; b. 3087 mm. Hg; c. 2311 mm. Hg; d. 1520 mm. Hg; e. 760 mm. Hg; f. 380 mm. Hg; g. 190 mm. Hg; h. 95 mm. Hg.

TABLE I

D. virilis: vertical amplitude of wingbeat as a function of air pressure and density

Pressure in mm. Hg	3863	3087	2311	1520	760	380	190	95
Density in gm. per L.	5.97	4.76	3.56	2.34	1.16	0.57	0.28	0.13
Specimen Number	Stroke Amplitude in Degrees of Arc							
516–3	102	103	112	112	127	129	—	137
517–1	100	103	109	98	117	138	138	138
517–2	100	92	96	103	111	127	149	143
517–3	80	99	105	90	111	106	121	138
518–2	104	113	105	102	118	127	124	121
518–3	110	111	113	128	123	139	134	141
518–4	102	114	114	119	124	126	132	134
518–5	99	114	120	130	131	144	147	129
519–1	94	113	116	121	148	148	152	158
519–2	114	124	117	127	135	154	137	139
619–2	119	121	108	106	124	132	140	142
Average	103	110	110	113	124	133	138	138
Standard Error	±3.1	±2.9	±2.1	±4.1	±3.3	±3.9	±3.2	±2.8
Number of Observations	11	11	11	11	11	11	10	11

Flight was permitted only during the making of a record. Each insect was first studied at normal pressure, after which the same individual was tested over the series of positive and negative pressures shown in Table I. Ordinarily only one or two records were taken at each pressure. The data reported in the several tables represent average figures in all cases where more than one record was available.

In analyzing the photographs, each record was projected and traced at about 8 diameters. The apparent amplitude, in head-on view, was then read from the drawing with the aid of a protractor, as the angle between the two extreme positions of the anterior margin of the wing.

Because of foreshortening in pictures taken in lateral view, the apparent position of the wing in such records may differ appreciably from the true position. The latter was therefore determined by reconstruction before measurements were made, as follows (see Fig. 4):

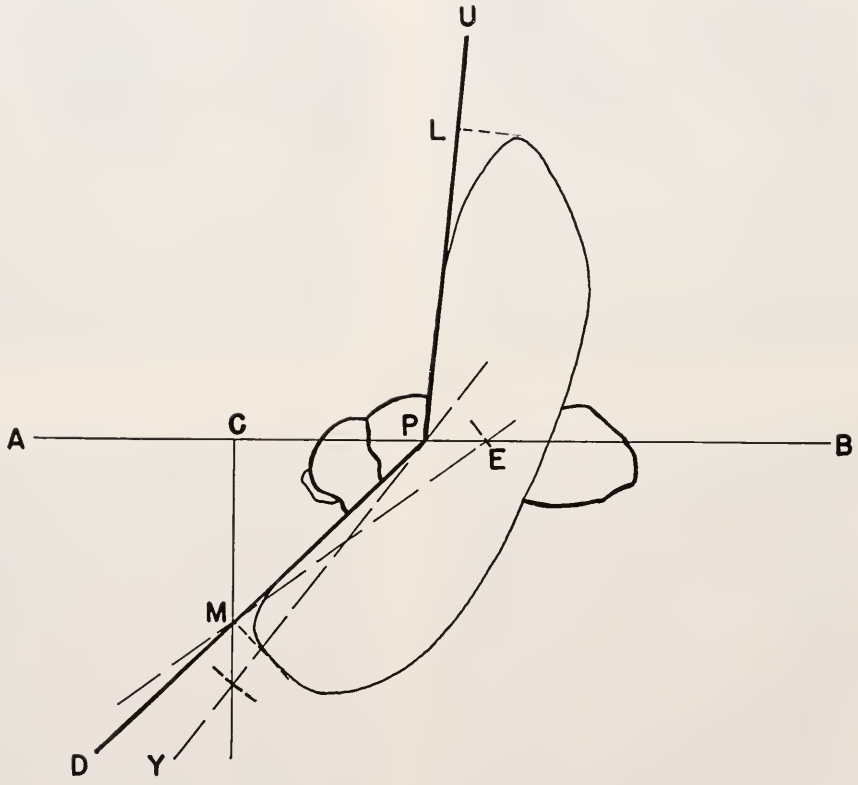

FIGURE 4. Diagram to show method of reconstructing from a photograph in side view the true angular displacement of the wing during the downstroke. For explanation, see text.

A horizontal line, AB, was drawn through the wing articulation, P, on the tracing; and extensions, UP and DP, of the costal margin in the extreme up and down positions were constructed. The true relative length of the wing, PL, was determined by dropping a perpendicular from the wingtip to UP in a record

of the same specimen taken at normal pressure, where it was known from head-on photographs that the costa is approximately in a vertical, parasagittal plane. The apparent length of the wing in the downstroke, PM, was similarly laid off; and a perpendicular, CM, drawn from M to AB. The true position of the costa in the downstroke was then found as the line connecting (1) P, and (2) the intersection of line CM with an arc of length PL centered at P. This reconstructed position of the costa is represented by the broken line, PY, in the figure.

Angles UPA and YPA were now recorded.

A similar reconstruction could be made for the position of the costa in the upstroke, at pressures other than normal, or in the downstroke in head-on view, but such corrections proved insignificant in practice and were therefore omitted.

TABLE II

D. virilis: variation in components of stroke amplitude as a function of air pressure and density

Pressure in mm. Hg	3863	3087	2311	1520	760	380	190	95	
Density in gm. per L.	5.97	4.76	3.56	2.34	1.16	0.57	0.28	0.13	
	Displacement Relative to Frontal Plane in Degrees of Arc								Type of Record
Upstroke	72	77	76	79	86	93	94	93	Front view
Downstroke	−31	−33	−34	−34	−38	−40	−44	−45	Front view
Downstroke	−34	−38	−35	−34	−38	−39	−47	−43	Side view
	Displacement Relative to Transverse Plane in Degrees of Arc*								
Upstroke	−5	−1	−4	−3	−7	−6	−5	−5	Side view
Downstroke	20	20	29	22	29	28	25	27	Side view

Each datum is the mean of from 6 to 30 measurements.

* Negative values here indicate displacement in a posterior direction.

Angle YPA gives a measure of the true forward displacement of the costa at the conclusion of the downstroke. The vertical displacement may also be obtained from the same record by drawing the line through M and the intersection, E, with the horizontal, AB, of an arc of length PL centered at M. The angle MEP is then a measure of the actual depression of the costa below the horizontal. Average values for angle MEP were obtained in this way from records of 6 animals which flew at all pressures. As indicated in Table II, such calculated values are in substantial agreement with the mean values measured directly from head-on pictures of other flies.

RESULTS

Table I records the total vertical amplitudes measured from head-on records of 11 specimens which, with one exception, flew at all 8 pressures tested. From the same records, the displacement of the costa above and below the horizontal was determined separately; the averages of these values are shown in Table II, which includes for comparison the average downward displacements obtained

from 6 sets of records in side view (see the preceding section for the method of measurement). Also shown in Table II is the average angular displacement of the costa in the fore and aft direction at the conclusion of up- and downstroke, as determined from photographs taken in side view. Other alterations in the envelope of the wing movement in response to density change are illustrated by the series of silhouettes in Figure 3.

Discussion

The data depict a significant trend toward greater amplitudes in the transverse plane at lower densities, and vice versa (Table I). When density is increased, both the upward and downward excursions of the wing are curtailed, while there is little change in the extreme fore and aft displacement of the wing (Table II). However, the trajectory of the wing varies in such a way as to give a broader envelope at lower densities when recorded in lateral view (Fig. 3).

The relationship between vertical displacement of the wing and density is linear on a double logarithmic scale, permitting calculation of the following regressions:

(a) limit of displacement above body axis (a_u) on density (ρ),

$$\log a_u = 1.9140 - 0.0885 (\log \rho - 0.2344); \quad (1)$$

(b) limit of displacement below body axis (a_d) on density (ρ),

$$\log a_d = 1.5688 - 0.0976 (\log \rho - 0.0944); \quad (2)$$

(c) total vertical amplitude (a) on density (ρ),

$$\log a = 2.0721 - 0.0934 (\log \rho - 0.2344). \quad (3)$$

In the calculation of equations (1) and (3), the empirical points at the lowest density, 0.13 gram per liter, were omitted, since they deviate significantly from the lines defined by the other observations. This discrepancy may be due to the fact that the upward displacement of the wing has already reached its physical limit, not greatly in excess of 90 degrees, at densities greater than 0.13 gram per liter. Another possibility is that performance at the lowest density was influenced to some extent by oxygen lack. In this series of experiments no attempt was made, as was done in the case of frequency (Chadwick and Williams, 1949), to rule out variation in oxygen tension and in total pressure as such as factors in the results obtained, since with the possible exception just noted, one sees no reason to suppose that either is implicated in the amplitude changes observed. But, in moist air at 27 degrees C. and at a density of 0.13 gram per liter the total pressure is 95 mm. Hg and the partial pressure of oxygen only some 15 mm., a value known to be close to the limit consistent with brief interrupted flight. It was frequently difficult to obtain a satisfactory response from the specimens at this pressure, and it is possible that those which flew were in a subnormal state. The remaining observations show but little scatter about the lines calculated, as judged by the chi-square test for dispersion. One may conclude then that amplitude is proportional, on the average, to the -0.09 power of density.

This finding may now be examined along the lines followed in the previous study of the dependence of wingbeat frequency on gas density.

$$P_o \propto mv^2f; \tag{4}$$

where P_o equals power output; m is the mass of air moved per stroke; v, the average velocity imparted to mass m; and f, wingbeat frequency. Or

$$P_o \propto V_s\rho v^2f, \tag{4a}$$

since m equals $V_s\rho$, where V_s is stroke volume and ρ, air density.

Now, it is apparent that amplitude is a factor in both the stroke volume, V_s, and the velocity of the wing, v_w. To the latter the air velocity, v, must, in turn, be proportional. Consider that an approximation of the stroke volume, in terms of the wing dimensions, is given by regarding the volume swept out by the wing as the segment of a cylinder with radius equal to the wing length, L, and with height equal to the product of mean wing width, W, and the sine of the angle of attack, α. Then

$$V_s \text{ (both wings)} = 2\pi L^2W \sin \alpha(2a/360°), \tag{5}$$

where a is the stroke amplitude. For a similar calculation of the mean velocity imparted to the air, it is necessary to take account of the shape of the wing, which is narrowest at the base. The mean velocity is therefore that produced by a unit area situated somewhat further than half the wing length from the articulation. Calling this radius L_m, we may then write

$$v \propto v_w \propto 2\pi L_m(2a/360°)f. \tag{6}$$

On substitution of expressions (5) and (6) for stroke volume and velocity, respectively, into equation (4a) above, one obtains:

$$P_o \propto a^3f^3\rho \sin \alpha. \tag{7}$$

Reference to the study by Chadwick and Williams (1949) shows that wingbeat frequency (f), for different individuals, varies on the average as the -0.09 power of density; that is, in the same quantitative manner as amplitude. By substituting $\rho^{-0.09}$ for both amplitude (a) and frequency (f) in equation (7), one finds

$$P_o \propto \rho^{0.46} \sin \alpha. \tag{8}$$

Provided then that no further factor of importance has been overlooked, one may judge that equilibrium can be maintained under constant power output only by variation of sin α as $\rho^{-0.46}$.

Reasons for supposing P_o constant, which were discussed fully in the previous publication, may be summarized as follows:

(a) P_o is equal to the product of power input, P_i, and an efficiency factor, e;

(b) P_i, measured in terms of oxygen consumption at different densities, is essentially constant;

(c) any factor which is to balance the relationship between frequency, amplitude, and density must show *continuous* exponential variation over the full range of densities investigated;

(d) a large increase in efficiency at higher densities, required if the relationship is to be balanced by alteration in P_o while P_i remains constant, is unlikely.

Although one is led by this reasoning to reject changes in efficiency as a plausible solution of the problem, it has been pointed out by O. Sotavalta (1947, 1951) that this treatment neglects energy losses due to inertia of the wing. These, he feels, may be considerable, inasmuch as the wing is reversing its direction of movement twice per cycle at frequencies of the order of 200 per second. Since there is appreciable variation in both frequency and amplitude, and hence, by inference, in rotational inertia of the wing, when density is altered, one must admit that the assumption of constant power output will be in error to the extent that efficiency is changed by variation in this loss. The possible significance of this factor in the flight of *D. virilis* may be assessed as follows:

$$E = 1/2\ I(\omega)^2; \tag{9}$$

where E is the mean kinetic energy of rotation of the wing at any instant; I, the rotational inertia of the wing; and ω, its mean angular velocity, *i.e.*,

$$\omega = 2\pi(2a/360°)f. \tag{10}$$

Now,

$$I = m_w r^2, \tag{11}$$

where m_w is the mass of the wing and r the radius of gyration, which may be taken as approximating $\sqrt{1/L \int_0^L r^2 \cdot dr}$, or $L \times 3^{-0.5}$, L being the wing length.

If E is dissipated twice per stroke, the power loss per wing is $2fE$; per insect,

$$P_e = 4fE. \tag{12}$$

Combining equations (9), (10), (11) and (12) gives the solution (on substitution of the measured average values, for *D. virilis*, of 2×10^{-1} cm. for wing length, L, and 3.625×10^{-6} gram for wing weight, m_w):

$$P_e = 1.1784 \times 10^{-10}\ \text{gm.cm.}^2 \times f^3 \times a^2. \tag{13}$$

From this equation, the values of P_e at different densities have been worked out, using our measurements of average f and a, and are given in Table III.

TABLE III

D. virilis: variation at different air densities of power loss due to inertia of wings

Density ρ grams per liter	Frequency* f cycles per second	Amplitude* a degrees of arc	Power Loss P_e ergs per second	Power Available for Useful Work P_0 ergs per second	$P_e/P_0 \times 100$ per cent
5.97	205	105	11.19	204.49	5.5
4.76	210	107	12.49	203.19	6.1
3.56	215	110	14.17	201.51	7.0
2.34	223	115	17.28	198.40	8.7
1.16 (1 atm.)	238	123	24.04	191.64	12.5
0.57	254	131	33.14	182.54	18.2
0.28	271	140	45.97	169.71	27.1
0.13	290	150	64.67	151.01	42.8

* Frequencies computed from data of Chadwick and Williams (1949); amplitudes, from equation (3) above; for full explanation of other calculations see text.

A figure for the *effective* power output at normal density, 25 degrees C., is available from unpublished data of Williams on *D. repleta*; the maximal value measured was 159.74 ergs per second. Discounting the species difference, since *D. virilis* and *D. repleta* have approximately the same size and flight characteristics, and allowing for a measured frequency increase of 421 beats per minute per degree C. (since $P_o \propto f^3$), we can estimate the effective power output at normal density under the conditions of our present experiments at 191.64 ergs per second. If wing inertia, amounting to 24.04 ergs per second, is a complete loss, this would represent a total output of 215.68 ergs per second. The fraction available for moving air at each density may now be calculated by subtracting from 215.68 ergs per second the corresponding calculated loss, P_e, due to inertia. Thus we obtain the values shown in column 5 of Table III. Two significant facts which emerge from these calculations are: (1) the power loss due to wing inertia in the flight of Drosophila probably does not exceed 45 per cent of the effective power output even under the most adverse conditions, and is normally much smaller than this; (2) although this loss varies in the proper manner, increasing at low densities and vice versa, and in proportion to $\rho^{-0.45}$, it is not of sufficient magnitude to provide balance in the relationship of equation (8) in the absence of variation in sin α.

A few additional comments are needed in reference to these calculations of the wing inertia. The values derived above may be somewhat low, since the wing trajectory includes a varying horizontal component (see Fig. 3) not contained in our formula for the angular velocity. Another inaccuracy stems from the fact that the true radius of gyration is, of course, unknown, though one doubts that it is greater than the length assigned, since the wing, though narrower, is appreciably more massive toward the base. Granting that possibly somewhat higher estimates should be given for P_e than those shown in Table III, this error is counterbalanced to some extent by the probability that the value cited for the effective power output is low. The figure quoted is merely the maximal output determined under a particular set of experimental conditions, and there is reason to believe that somewhat greater outputs might be measured with improved techniques. Finally, the assumption that the kinetic energy of the wing becomes a total loss, twice per cycle, is open to question. A portion of this energy must be expended against the air and contribute useful work. Although the larger fraction in each half cycle is dissipated, no doubt, in stretching the antagonistic sets of muscles, one must reckon also with the possibility, given the very short duration of the cycle, that a proportion of even this fraction is reabsorbed (cf. Hill, 1950). Some energy may also be recaptured through elastic deformation of the thoracic cage. Weighing these various considerations together, it seems improbable that the actual loss of power due to wing inertia amounts to a fraction of the power output significantly greater than indicated in Table III.

We are thus left with a choice of assumptions: either the overall efficiency of flight varies, in some manner as yet unexplained, about as the -0.4 power of density, or the angle of attack of the wings on the air changes in the same proportion. Believing that most animals are fairly well adjusted to their normal environment, one is reluctant to postulate increases of efficiency amounting to something over 100 per cent when Drosophila are placed in the wholly unnatural situation of being forced to fly at an air pressure of 5 atmospheres. For this

reason the alternative, a comparable increase in the angle of attack at 1/8 atmosphere and an equivalent reduction at 5 atmospheres, seems the more acceptable, although we regret our inability to settle the question experimentally. If, as suggested by the silhouettes of Figure 3, the quantity $V_s\rho v^2$ varies somewhat more rapidly with changing density than has been estimated on the basis of our measurements of frequency and vertical amplitude, the actual rate of variation required in the angle of attack may be even less than we have been led to postulate above. It is to be hoped that methods for attack upon the several unknowns still remaining in this problem will be developed.

The writer is indebted to Dr. C. M. Williams for valuable discussion in regard to all phases of this investigation.

Summary

The amplitude of wingbeat of *Drosophila virilis* Sturtevant in moist air at a temperature of 27 ± 1 degrees C. and at a series of gas pressures ranging from 3863 mm. Hg to 95 mm. Hg, was recorded photographically.

The vertical amplitude of the wingbeat decreased progressively with increase in air pressure. The excursion of the wing was curtailed in the same proportion at both extremes of the stroke. These changes are attributed to alterations in atmospheric density which resulted from the change in gas pressure. It is calculated that amplitude varies approximately as $\rho^{-0.09}$.

No significant change was observed in the limits of angular excursion of the wing in a fore and aft direction, although the envelope of the wing movement, as seen in lateral view, broadened as pressure was reduced.

It is shown that the measured changes in amplitude and frequency compensate only in part for the change in load which results from alterations in air density. The possible role of power losses due to wing inertia is discussed, and it is concluded that these represent only a minor factor in the observations. Arguments are presented which suggest that alterations in gas density provoke compensatory changes not only in wingbeat frequency and stroke amplitude, but also in the angle at which the wings attack the oncoming stream of air. The degree of compensation required would be provided by doubling the angle of attack at 95 mm. Hg and halving it at 3863 mm. Hg.

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THE SOURCES AND ACTIVITIES OF TWO CHROMATOPHOROTROPIC HORMONES IN CRABS OF THE GENUS SESARMA. I. EXPERIMENTAL ANALYSES [1]

MASASHI ENAMI

College of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama, Japan

As is well known (Brown, 1944; Hanström, 1947, etc.), the significance of the sinus gland as the principal source of chromatophorotropic hormone or hormones has been well substantiated by investigations, done with a large number of species in a taxonomically wide range of crustaceans since the first description of this incretory tissue by Hanström (1933). On the other hand there is a growing knowledge of the possible presence of certain hormone sources outside the sinus gland which is owed chiefly to recent continued efforts by Brown and his associates (1940–1947). They have demonstrated hormone production within the central nervous organs first in Crago, and are extending the research to other kinds of crustaceans. Thus, in the light of present information, we may infer that chromatophorotropic hormones that are directly concerned with the physiological coordination of pigmentary effector systems of crustaceans are roughly classified into two broad categories, *viz.*, the one originating in the sinus gland and the other in the nervous tissues.

Nevertheless, we are as yet only fragmentarily informed of details in connection with the actual number of hormones belonging to these respective categories, the essential activity of each of the hormones, the mode of interaction among them, etc., which is still keeping us from postulating certain generalizations with respect to the endocrine mechanism for the control of crustacean chromatophores.

Having been engaged in physiological as well as histological analyses of endocrine organizations in crabs of the genus Sesarma, with special reference to the production of chromatophorotropic hormones, I obtained some results which may furnish information concerning some of the pending problems just mentioned.

Materials and Methods

The animals used in the physiological analysis of the endocrine control of chromatophores were three representatives of the genus Sesarma, *viz.*, *S. intermedia*, *S. haematocheir* and *S. dehaani*, the most familiar brackish-water crabs in Japan. Adults of the respective species weighing ca. 30 g. (males) or ca. 25 g. (females) collected in the field were kept alive in the laboratory until required for experimentation.

For the assay of chromatophorotropic hormones present in the crabs, tissues and organs were dissected out from individuals showing no sign of ecdysis. In

[1] The present work was supported by a grant-in-aid from the Ministry of Education of Japan during the fiscal years of 1947 and 1948. Research facilities were provided by the Mitsui Institute of Marine Biology, Shimoda, Japan, where the work was carried out.

order to reject specimens in the molting phase, the early indication of the phase at the epipodite of the first maxilliped (cf. Drach, 1939) was resorted to. Isolated tissues and organs were separately ground with quartz sand to be extracted twice with distilled water. The insoluble fraction was centrifuged off, and the supernatant fluid was sealed in a glass tube and dipped into boiling water for 10 minutes. Such a preparation could be preserved for weeks and months without significant loss of hormone activity. When needed for the assay, the samples were evaporated *in vacuo* to be re-extracted with physiological saline. The final extracts were freed by filtration from coagula formed in the process of previous heating. Concentrations of extracts were expressed in terms of the number or weight of the respective tissues or organs theoretically contained in the administered doses, which were designated as T.E. (tissue equivalents). The chromatophorotropic activity of the extracts was tested chiefly upon the chromatophores of juvenile forms of *Sesarma haemato-*

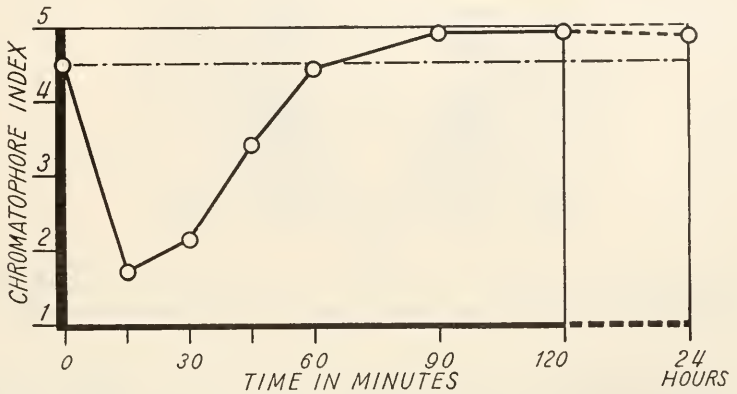

FIGURE 1. Curve showing time relation of pigmentary response of black chromatophores of juvenile *S. haematocheir* following amputation of both eyestalks.

cheir with carapace dimensions less than 20 mm. × 18 mm. Such individuals, in spite of the marked sluggishness in the chromatic display of adult forms of the three species of Sesarma, show a high chromatic activity, changing coloration from creamy yellow to chestnut brown uniformly over the whole dorsal aspect of the body including the walking legs. Background responses are lacking like in other color-changing crabs, and, under laboratory conditions during the day, the immature crabs exhibit darkening that is considerably subject to fluctuations of environmental factors such as the intensity of light, moisture and heat.

Upon bilateral amputation of the eyestalks, remarkable blanching of short duration takes place, which is followed by a process of re-darkening, and the eyestalkless animal eventually attains maximum darkness of body shade that is regarded to be the equilibrium state of pigmentary activity in the day, characteristic of the eyestalkless condition. Mainly responsible for such macroscopic changes of coloration are the brownish black chromatophores (Fig. 1), but the animal is provided with additional chromatophores, *viz.*, the red, the vermilion and the white cells, which, though limited in the chromatic effects, are also significant for the pigmentary behavior.

In the equilibrium state of the pigmentary activity of the eyestalkless animal, the black chromatophores are seen in a state of maximum pigment dispersion, the red and the vermilion cells together are in states of approximately semi-dispersion, and the white ones are in the state of nearly maximum concentration. Such states of the chromatophores were used *in vivo* and *in vitro* as test objects for the assay of the chromatophorotropic effectiveness of the extracts prepared from adult Sesarma.

Tissue or organ extracts re-extracted with known amounts of van Harreveld's solution buffered to pH 7.5 (mean pH value of the body fluid of Sesarma in the intermolt phase) were administered to the test animals that had been eyestalkless for at least two days, by injection into the thoracic musculature through the arthrodial membrane at the basal articulation of the second walking leg. Five one-hundredths cc. was injected per individual at a time; a lot of 15 test animals was employed for one assay.

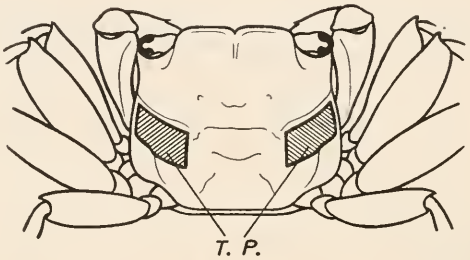

FIGURE 2. Dorsal view of juvenile *S. haematocheir*. Areas hatched (T. P.) at mesobranchial regions of dorsal carapace used as test pieces for supravital experimentation.

Extracts were tested also upon the chromatophores in isolated fragments of carapace, obtained from the mesobranchial region of the eyestalkless juvenile *S. haematocheir* (Fig. 2). As was already remarked by Brown and Meglitsch (1940), chromatophores surviving in isolated carapace fragments usually show a considerably lowered reactivity, and significant pigmentary displays are often brought about by only those distributed at the periphery of each fragment. In the present experiments, however, experience taught that frequently repeated rinsing with van Harre-

TABLE I

Size and density of four types of chromatophores in mesobranchial region of juvenile S. haematocheir of carapace dimensions of 16 mm. × 14 mm. Measurements upon eight individuals

Chromatophore / Size and Density	Black	Red	Vermilion	White
Optical Diameter at Maximum Pigment Concentration	33μ–55μ commonly ca. 38μ	11μ–33μ commonly ca. 22μ	11μ–22μ commonly ca. 17μ	15μ–33μ commonly ca. 22μ
Number in 1 mm.² of Carapace Area	87–165	6–28	74–174	96–252
Mean ± σ	120.4 ± 21.1	18.6 ± 6.9	117.9 ± 29.7	166.0 ± 54.8

veld's solution of carapace fragments directly following removal from the body was fairly good for maintaining the reactivity of chromatophores, so that use was made of carapace fragments washed for one hour in the solution that was renewed every 15 minutes. Such a treatment did not seriously affect the initial pigmentary conditions of chromatophores in the eyestalkless animal; responsiveness of the latter was preserved for an additional 10 hours under 25°C. In the assay of the extracts, 10 pieces of carapace fragments of 1 mm. × 2 mm. obtained from 10 different eyestalkless individuals were immersed in 3 cc. of the respective extract. The useful idea of T.E. was adopted too for the expression of concentrations of extracts.

TABLE II

Table summarizing chromatophorotropic activities of extracts from adult Sesarma as tested by injection upon Paratya, Ocypoda and Megaligia

Test Animal		*Paratya* Eyestalkless			*Ocypoda* Eyestalkless				*Megaligia* "Blinded"	*Megaligia* "White-adapted"
Chromatophore		Red	Yellow	White	Black	Red	Orange	White	Black	
Initial Pigmentary State		D	D	S–D	C	D	D	S–D	D	C
Effect of Injection of Extract	Sinus Gland	C	C	D?	D?	C	C	D	C?	D
	Eyestalk excluding Sinus Gland	C	C	C	D?	C	C	D		
	Optic Ganglia	C	C	D?					—	D?
	Medulla terminalis	C	C	C	D?	C	C	D	C?	D
	Brain	C	C	C	D?	C	C	D	C?	D
	Commissural Ganglion	C	C	D?					—	D?
	Thoracic Ganglion	C	C	D?	D?	C	C	D	C?	D

Notations: C, pigment concentration; D, pigment dispersion; S–D, semi-dispersion of pigment; ?, indefinite pigmentary response; —, no visible pigmentary response.

Extracts of Sesarma were further tested upon the chromatophores of *Paratya compressa,* a fresh-water shrimp, *Ocypoda stimpsoni,* a sea-shore crab, and *Megaligia exotica,* a sea-shore isopod, which all show remarkable color change. Concerning the physiology of color change of Paratya and Megaligia, papers by Nagano (1943) and Enami (1941) are to be referred to. For the present purpose, eyestalkless Paratya, eyestalkless Ocypoda, and "blinded" (eyes covered with a mixture of Sudan Black and balsam) and "white-adapted" (eyes covered at the dorsal aspect with the opaque mixture, and the animal kept in white container under dim light) Megaligia were employed. Equilibrium states of chromatophores in each of these test animals are summarized in the upper column of Table II. Van Harreveld's solution was employed as the physiological solution for Paratya, and Herbst's arti-

ficial sea-water for Ocypoda and Megaligia. The dose of injection was 0.01 cc. for Paratya, 0.05 cc. for Ocypoda and 0.02 cc. for Megaligia. Fifteen individuals of each species composed one lot of test animals.

In all these experiments, chromatophore responses *in vivo* or *in vitro* were observed at intervals of 15 minutes following administration of extracts of Sesarma, and were recorded by means of the conventional chromatophore indices. For the application of the indices to actual pigmentary states of chromatophores in a given area of the skin, the arbitrary method proposed by Hogben and Slome (1931) was adopted. Indices obtained with a lot of test objects at a given time were averaged to be plotted for a graph representing the time relation of chromatophore responses. This indicated the effectiveness of a given extract.

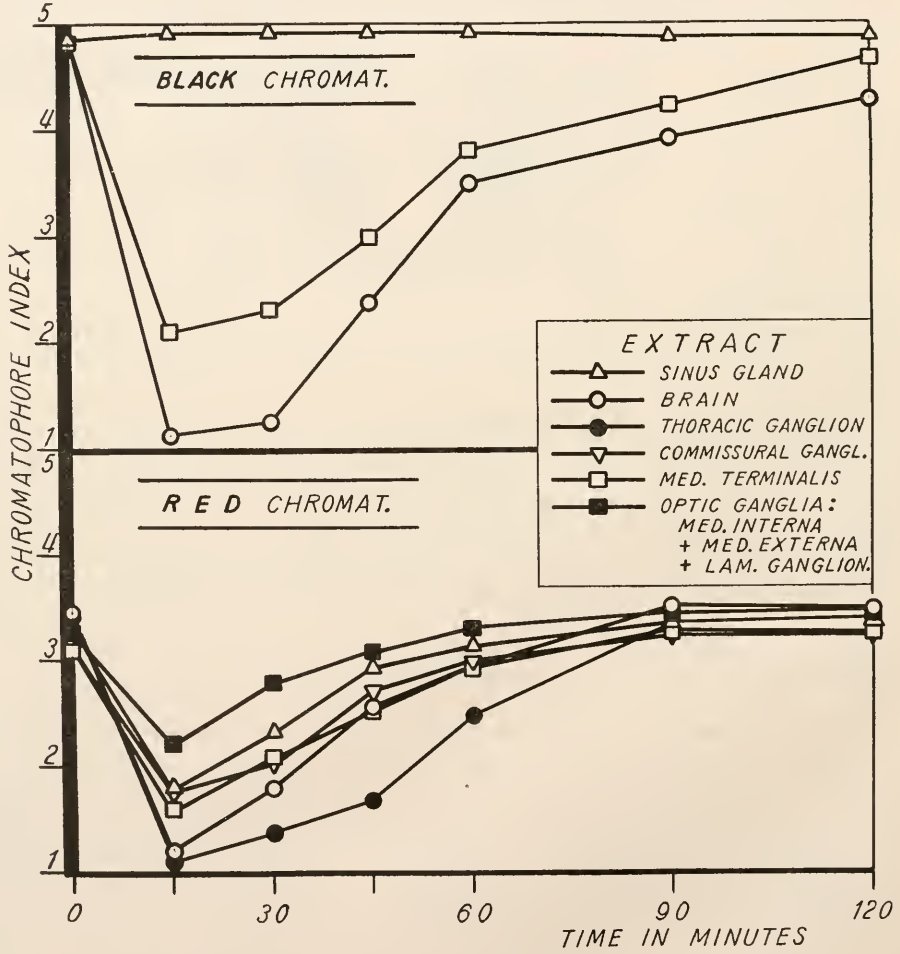

FIGURE 3. Curves showing time relations of responses of black and red chromatophores of eyestalkless *S. haematocheir*, juv. to injection of active extracts from adult *S. dehaani*. Concentration was 0.5 T.E. per 0.05 cc. in respective extracts.

Experiments and Results

Because it was concluded that in the three species of Sesarma employed the endocrine system is alike, for the sake of simplification the following account is generalized and covers all species used.

Injection Experiments upon S. haematocheir, juv.[2]

The result of injection experiments with tissue and organ extracts from adult forms of Sesarma was that only the extracts of sinus gland and nervous organs were effective in inducing definite chromatophore responses in the eyestalkless *S. haematocheir,* juv. Others, such as the extracts of stomach, intestine, hepatopancreas, heart, male genitalia, gill, muscle and hypodermis together with underlying connective tissue, were not responsible for any definite chromatophore reaction, neither was the control injection of plain van Harreveld's solution.

From the point of view of qualitative effects (cf. Fig. 3), the activities of the extracts might be arranged according to the following classification:

a) Extracts to which the red and the vermilion chromatophores were susceptible. Response: correlated pigment concentration in both chromatophores. Extracts: sinus gland, Lamina ganglionaris, Medulla externa, Medulla interna, Medulla terminalis, brain, commissural ganglion, thoracic ganglion.
b) Extracts to which the black and the white chromatophores were susceptible. Responses: pigment concentration in black chromatophores, being correlated with pigment dispersion in the white ones. Extracts: brain, Medulla terminalis.

In repeated trials of dilution experiments, the ineffectiveness of the sinus gland extract for the black and the white chromatophores was proved over a concentration range reaching as high as 5 T.E. per 0.05 cc., while a more or less significant effect was detected for the red and the vermilion cells even in a concentration as low as 0.001 T.E. per 0.05 cc. Extracts of all the principal nervous organs including brain and Medulla terminalis were effective for the red and vermilion chromatophores in various degrees, those of larger ganglia, such as thoracic ganglion, brain and Medulla terminalis, being even more effective than the sinus gland extract in the same T.E. The black and white chromatophores were affected only by extracts of brain and Medulla terminalis, the former extract exceeding the latter in effectiveness, acting even in a concentration of 0.0015 T.E. per 0.05 cc. Figure 4 shows the relation between the concentration of the brain extract and the magnitude of response of the black chromatophores, measured from time relation curves obtained by a series of dilution experiments. As is shown, the magnitude of effectiveness was exponentially proportional to the concentration of the extract, the relation between them being significantly sensitive only over a restricted range of lower concentrations.

Experiments upon Isolated Carapace Fragments of S. haematocheir, juv.

From the experiments with isolated carapace fragments obtained from eyestalkless *S. haematocheir,* juv., the qualitative activities of the active extracts of adult Sesarma were again ascertained. Observations with the carapace fragments turned

[2] Abbreviation of juvenile form.

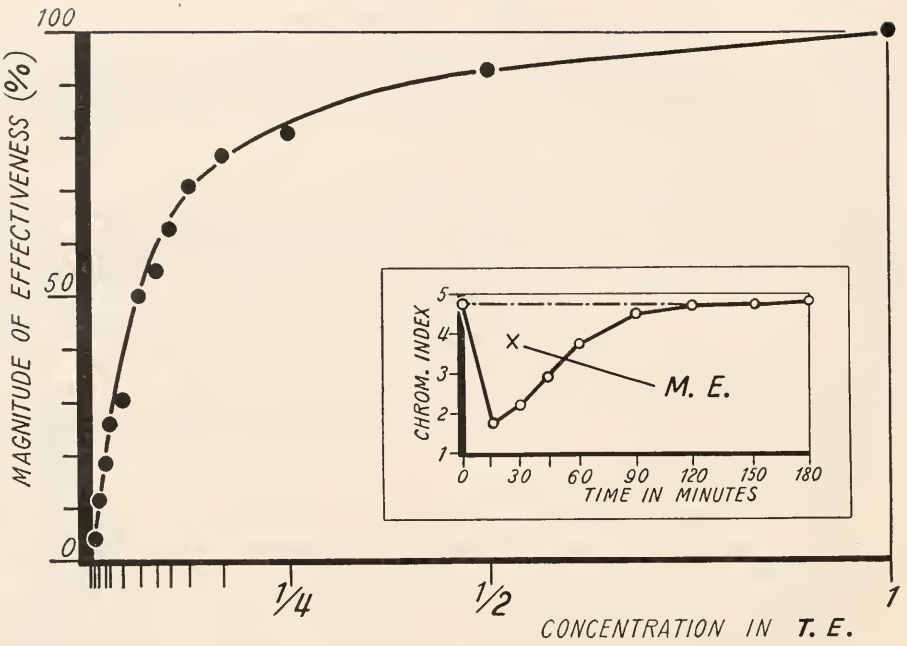

FIGURE 4. Curve showing relationship between percentage of magnitude of effectiveness and concentration of brain extract from adult *S. dehaani*. Figure inserted indicates method of estimation of magnitude of effectiveness: Area enclosed by time relation curve of response of black chromatophores and a horizontal line representing initial pigmentary state of the chromatophores was measured and designated to be magnitude of effectiveness (M.E.) of given extract. M.E. of 1 T.E. per 0.05 cc. was taken as standard value, against which percentile ratio of M.E. of respective concentration was calculated.

inside out facilitated detailed examination of the behavior of the white chromatophores, which are located in the deepest layer of the skin and are masked in various degrees when viewed from the outer surface of the carapace. The correlated responses of the cells with the black ones under the influence of the extracts of brain and Medulla terminalis were clearly demonstrated in the course of the experiments (Fig. 5).

Hormone Production by Nervous Organs

The foregoing results indicated that a kind of chromatophorotropic hormone, or a group of hormones, which, with respect to its qualitative effect, appeared to be identical with the one included in the sinus gland (sinus gland hormone), was almost universally distributed in the central nervous system of adult Sesarma. The question arose whether such a kind of hormone was originating essentially in the nervous tissue or whether it was derived from the sinus gland and stored in the former.

The question seemed to be answered in part by the fact that the effectiveness of extracts of some ganglionic tissues exceeded that of the sinus gland extract, which fact might suggest that the hormone under consideration was more concentrated in certain ganglia than in the sinus gland itself. However, definite proof of the

origin of the hormone in the nervous tissues themselves was obtained from an experiment, designed for the assay of the activity of the brain extract prepared from a lot of adult Sesarma that had been kept in the eyestalkless state for 30 days. Since the donor of the extract had been without the sinus gland, the brain extract might be reasonably concluded to be free from possible contamination of the sinus-gland hormone.

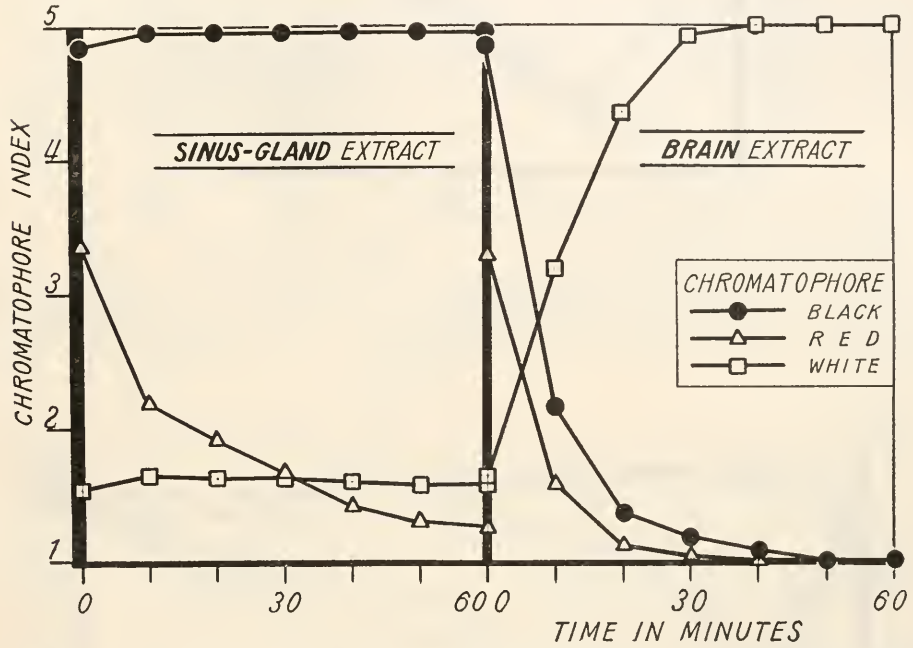

FIGURE 5. Curves showing time relations of responses of black, red and white chromatophores from eyestalkless *S. haematocheir,* juv., kept *in vitro,* to application of active extracts from adult *S. intermedia.* Concentration was 3 T.E. per 3 cc. in respective extracts.

As is shown in Figure 6, the effectiveness of the extract for the red and the vermilion chromatophores was almost comparable to that of the extract prepared from the normal animals. The same was the case with the effectiveness for the black and the white chromatophores, an observation which indicated the possible independence of the mechanism for the production of the respective hormones within the brain.

Hormone Production in Juvenile Forms of Sesarma

Injection experiments as well as experiments upon isolated carapace fragments were carried out with extracts prepared from juvenile forms of the three species of Sesarma, using the chromatophores of *S. haematocheir,* juv. as test objects. The result was that the chromatophorotropic principle known to be present in the nervous system of adult crabs was also found in juvenile forms. As to the distribution of the hormone affecting the black and the white chromatophores, it ap-

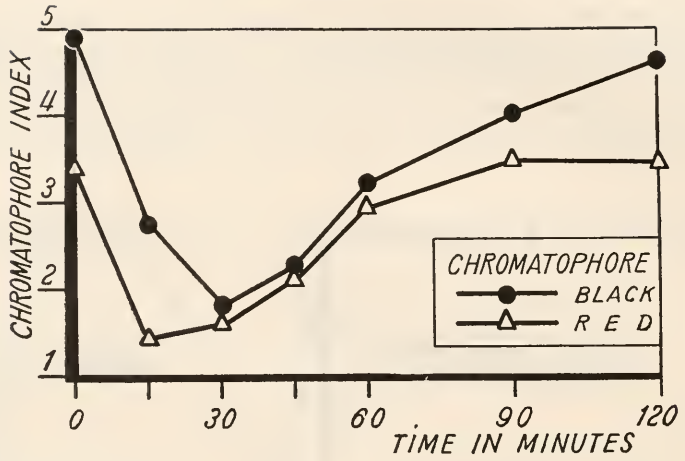

FIGURE 6. Curves showing time relations of responses of black and red chromatophores of eyestalkless *S. haematocheir*, juv. to injection of brain extract from adult *S. dehaani* that had been kept in eyestalkless state for 30 days. Concentration of extract was 0.5 T.E. per 0.05 cc.

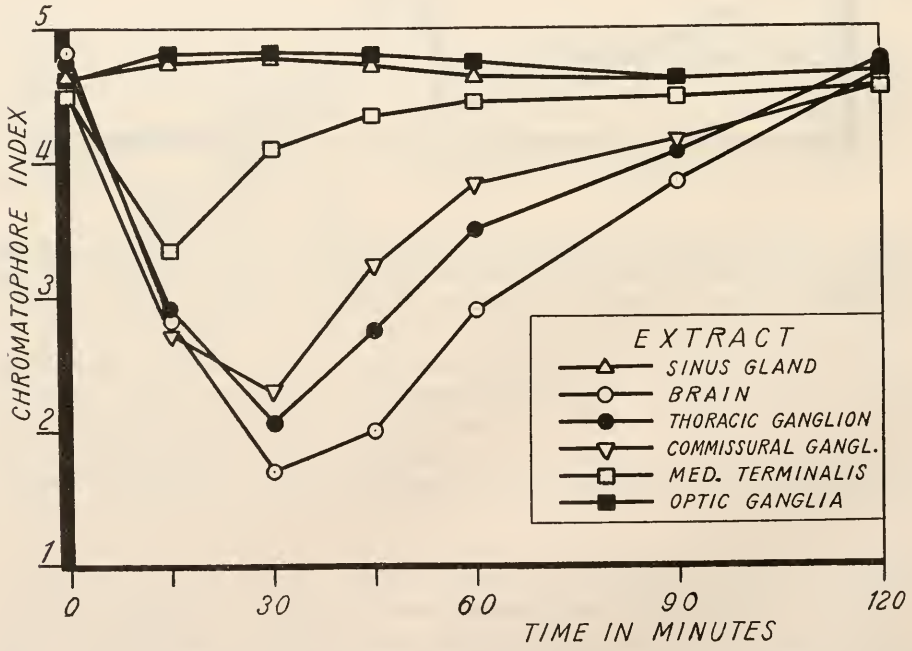

FIGURE 7. Curves showing time relations of responses of black chromatophores of eyestalkless *S. haematocheir*, juv. to injection of active extracts from juvenile *S. dehaani*. Concentration was 1 T.E. per 0.05 cc. in respective extracts.

pears to be distributed over a larger area of the nervous system in juvenile specimens than in adults, *i.e.*, in young forms not only the brain and the Medulla terminalis, but also the thoracic and commissural ganglia rendered active extracts (Fig. 7). The order of effectiveness of these extracts for the black chromatophores in the same T.E. was: brain > thoracic ganglion > commissural ganglion ≫ Medulla terminalis.

The observation that a certain incretory mechanism for the production of a kind of hormone, responsible for pigment concentration in the black chromatophores, was detected also in juvenile forms of Sesarma, suggested an explanation for the underlying mechanism of the blanching response occurring upon amputation of the eyestalks in *S. haematocheir*, juv. (cf. Fig. 1, and the account in MATERIALS AND METHODS).

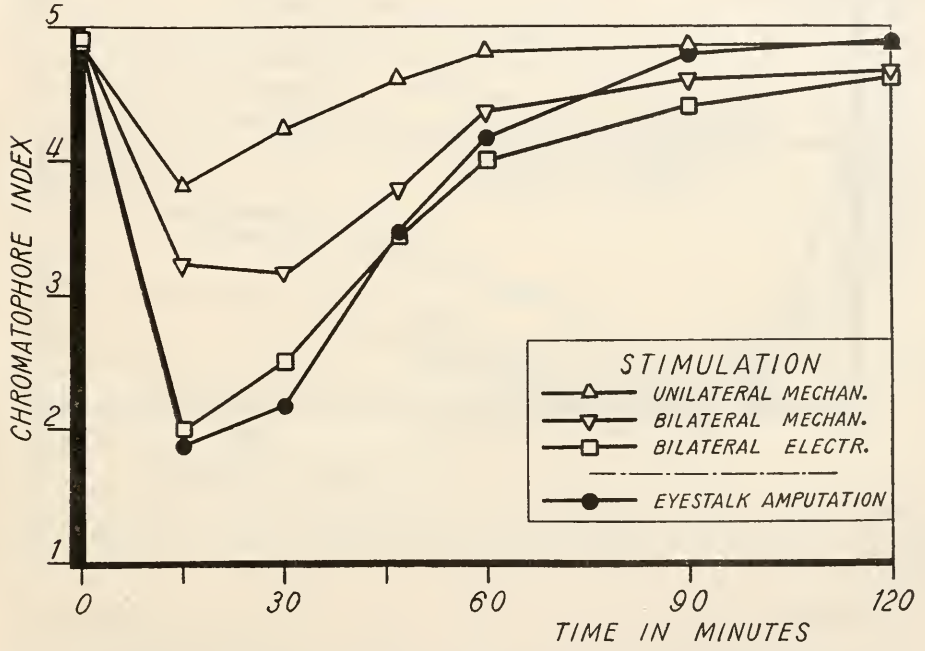

FIGURE 8. Curves showing time relations of responses of black chromatophores of eyestalkless *S. haematocheir*, juv. subjected to stimulation of stub of eyestalk.

A similar temporary blanching response was brought about by means of bi- or uni-lateral stimulation of the eyestalk-stubs of eyestalkless *S. haematocheir*, juv. (Fig. 8). Stimulation was performed either mechanically (pressing with small spherical head of a slender glass rod) or electrically (applying faradic current induced by the Harvard Inductorium connected to a 2 volts source; employing the bi- or uni-polar method). A comparison of time relation curves of reactions of the black chromatophores to respective treatments with that obtained as the result of amputation of eyestalks, indicated that all the reactions occurred under similar circumstances in the immediate surrounding of the chromatophores. In other words, it may be concluded that stimulation of the optic tract activates an incretory mechanism of the

brain or other ganglia to deliver the hormone responsible for pigment concentration in the black chromatophores.

Regarding such possible nervous control of hormone secretion by the nervous tissues, an attempt was made to inquire into the effect of acetylcholine, pilocarpine and atropine. These were injected in doses ranging from 2.5 γ to 50 γ, but none of them was responsible for any positive effect (*i.e.*, pigment concentration in the black chromatophores).

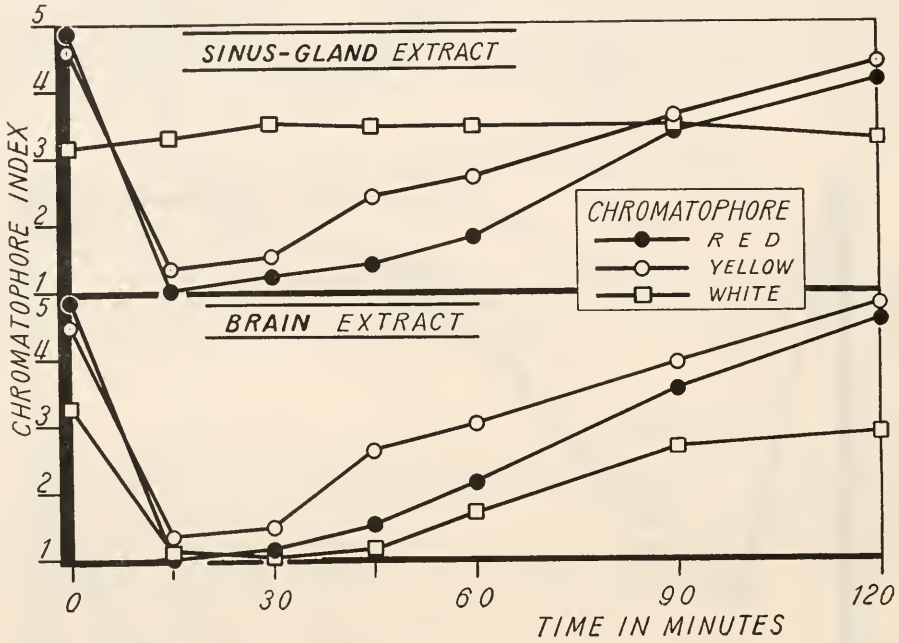

FIGURE 9. Curves showing time relations of responses of red, yellow, and white chromatophores of eyestalkless *Paratya compressa* to injection of active extracts from adult *S. intermedia*. Concentration was 0.5 T.E. per 0.01 cc. in respective extracts.

Mutual Independence of Peripheral Actions of the Two Kinds of Hormones

Assuming on the basis of the foregoing results that the sinus gland contains a hormone affecting the red and the vermilion chromatophores of *S. haematocheir,* juv., while the brain, besides containing a principle like that of the sinus-gland, also furnishes a hormone acting on the black and white cells, double injections of these extracts were tried for the purpose of learning something about any possible interference between the peripheral actions of the two hormones.

At first one of the extracts was injected, and at the end of 5 minutes the second injection of the other extract followed. In another series of experiments mixtures in various proportions of the two extracts were applied to isolated carapace fragments maintained *in vitro*.

In no case was there any significant antagonism between the two extracts. The response of the black chromatophores was always dependent upon the concentration of the brain extract, being by no means significantly affected by the co-existing

sinus-gland extract. The magnitude of response of the red and the vermilion cells was variously amplified in every instance over the range determined by the sinus-gland extract alone. This may be explained by possible summation of the effects of the hormone responsible for the red and vermilion cells present in both of the extracts applied, on the assumption that the hormone originating in the brain is identical with the sinus-gland hormone at least as to the peripheral activity.

Chromatophorotropic Activities of Extracts from Sesarma as Tested upon Paratya, Ocypoda and Megaligia

In order to obtain further information concerning the possible differentiation into two kinds of chromatophorotropic hormones in Sesarma, the activities of the effective extracts from adult forms were assayed upon the chromatophores of eyestalkless Paratya, eyestalkless Ocypoda, and "blinded" and "white-adapted" Megaligia. Results of injection experiments are summarized in Table II.

The table shows that the red and the yellow chromatophores of Paratya were influenced by the hormone widely distributed in the sinus gland as well as in all of the principal nervous organs of adult Sesarma, while the white cells were under the exclusive control of another kind of hormone originating in the brain and Medulla terminalis (cf. Fig. 9 also). However, the chromatophores of Ocypoda and Megaligia did not exhibit qualitatively different responses to the administration of different kinds of hormones of Sesarma.

Discussion

So far as the principal results of the present study are concerned, the endocrine systems of *Sesarma intermedia, S. haematocheir* and *S. dehaani* are to be classified into two categories on the basis of the distribution of the possible sources of chromatophorotropic hormones. One is represented by the sources of a hormone or a group of hormones which is responsible for correlated pigment concentration in the red and the vermilion chromatophores of *Sesarma haematocheir,* juv., and in the red and the yellow chromatophores of *Paratya compressa.* Such a kind of hormone is characteristic of the sinus gland, the universally admitted significant source of chromatophorotropic hormone, but, in Sesarma, a similar hormone is originating in all the principal nervous organs too, which fact is borne out by the wide distribution in the nervous systems of the crabs of certain incretory elements assumed to produce the active principle comparable to that of the sinus gland. Assuming tentatively the identity of the hormone originating in the nervous tissues with that in the sinus gland, I should like to propose the name S hormone (hormone characteristic of sinus gland) which comprises both the sinus-gland hormone and the one from the nervous tissues in the present cases.

The other endocrine system is represented by sources of a hormone responsible for correlated pigmentary reactions in the black (pigment concentration) and the white (pigment dispersion) chromatophores of *S. haematocheir,* juv. and pigment concentration in the white chromatophores of Paratya. In contrast to the wide distribution of the possible sources of S hormone, the range of distribution of the possible sources of this second kind of hormone is limited to particular portions of the nervous system, *i.e.,* brain and Medulla terminalis in adult forms, and brain, Medulla terminalis, thoracic and commissural ganglia in juvenile forms. In view

of the restriction of its possible source in the nervous tissues, I wish to designate such a hormone under the name N hormone (hormone essentially originating in the nervous tissues).

That a type of hormone, whose effect is qualitatively identical with the sinus-gland hormone, arises from the nervous tissues was already implied by Brown (1933), Hosoi (1934), Enami (1943), etc., who demonstrated certain measures of chromatophorotropic activity in extracts of various parts of the central nervous systems of different crustaceans. With *Uca dubia,* Enami proved that production of such a hormone in the brain and the thoracic ganglion is independent of the activity of the sinus gland, and, as already demonstrated, such is also the case with the present Sesarma.

Brown and Ederstrom (1940) offered the first concrete evidence with Crago of the occurrence in certain portions of the nervous system of a kind of hormone which is different from the sinus-gland hormone in its qualitative effect. A hormone which has thereafter been dealt with as CDH (Crago-darkening hormone) was at first detected in maximum concentration in extracts of the commissural ganglion, but later works by Brown and Wulff (1941) and Brown (1946) determined the site of essential source of this hormone to be the tritocerebral commissure. This CDH is said to be responsible for pigment dispersion in the black chromatophores distributed at the telson and uropods of Crago. Following the discovery of CDH, Brown and his associates (Brown and Wulff, 1941; Brown and Klotz, 1947) were successful in detecting and separating a second chromatophorotropic hormone (CBLH, Crago body-lightening hormone) originating likewise in the tritocerebral commissure, which is responsible for pigment concentration in the black chromatophores of the body except the telson and uropods. Antagonism was proved between these two kinds of hormones; in the absence of CBLH, CDH is effective in inducing pigment dispersion of the black cells over the whole body surface. Using Crago as test animal, Brown and Saigh (1946) examined the comparative distribution in the nervous systems of CDH and CBLH in 13 species representing Isopoda, Natantia, Astacura, Anomura and Brachyura, obtaining positive results with most of the animals. According to these authors, CBLH has in most cases wider distribution in the nervous systems as compared with CDH, which is rather restricted to certain particular portions of the nervous organs.

From the point of view of qualitative effect, what is designated as CBLH appears to be identical with the sinus-gland hormone, whose activity upon Crago black chromatophores has been determined since the pioneering work by Koller (1928). The identity of these hormones seems to be further substantiated by the work of Brown and Ederstrom (1940) who remarked that CDH and the sinus-gland hormone were mutually antagonistic. Further, the work by Brown and Klotz (1947) demonstrated that CBLH was considerably soluble in organic solvents, such as ethyl, methyl and isopropyl alcohols, in contrast to CDH, which had no significant solubility in these solvents, which fact appears to point out the similarity in chemical properties of CBLH to the sinus-gland hormone which has been demonstrated to be also considerably soluble in alcoholic solvents (Carlson, 1936; Abramowitz and Abramowitz, 1938; Abramowitz, 1940; Brown and Scudamore, 1940).

Such a consideration seems to permit a tentative generalization stating that CBLH together with the sinus-gland hormone may be dealt with under the same

category of chromatophorotropic hormones, which apparently corresponds to the present S hormone of Sesarma. Such a discussion appears to be premature in the present report, but it is tenable in that certain histological evidence proving the identity of secretory behaviors of the sinus gland and incretory elements found in the nervous systems was obtained with Sesarma, which will be published in a subsequent paper.

Assuming now that the nervous systems of crustaceans are generally charged with two kinds of chromatophorotropic hormones, as already postulated and proved by Brown, the present N hormone in Sesarma might be conceded to correspond to the said CDH, notwithstanding that CDH was reported to be absent in several brachyurans, such as *Libinia* sp., *Cancer irroratus, Carcinides maenas, Ovalipes ocellatus* and *Uca pugilator* (Brown and Saigh, 1946). As stated above, no significant antagonism was detected between S and N hormones when assayed upon the chromatophores of *S. haematocheir,* juv., but an inquiry into mutual interaction of these hormones is desirable upon the black chromatophores of Crago, before implied disparity of N hormone from CDH is taken into consideration. Brown and Saigh reported a differential distribution of CDH in the nervous systems of the crustaceans examined. In Sesarma, the distribution of N hormone in the nervous organs differs between the adult and the juvenile forms, being restricted in the former.

Hitherto no definite information was published as regards the production of chromatophorotropic hormones in the nervous tissues included in the eyestalk. Present investigation disclosed the universal occurrence of S hormone in the ganglia of the eyestalk of Sesarma and the restricted distribution of N hormone in the Medulla terminalis. On the basis of such knowledge, the data presented by Brown (1940) indicating the presence of considerable activity in the rest of the eyestalk tissues outside the sinus gland are to be attributed to the possible presence of S hormone in the ganglionic tissues in the eyestalks of crustaceans examined, *viz.,* Uca, Carcinus, Pagurus, Palaemonetes, Libinia, Callinectes and Crago. Further, Brown (1940) reported that the white chromatophores of Palaemonetes were, unlike the red ones, not significantly susceptible to the sinus-gland extract, which, according to an earlier paper by the same author (1935), were reported to be affected by the extract of the whole eyestalk. This fact appears to be explained on the assumption that N hormone is included in certain parts of ganglionic tissues in the eyestalk of the shrimp.

Thus evidence has been accumulating toward confirmation of the presence of two kinds of chromatophorotropic hormones in crustaceans, and it is to be added here that in Cambarus too McVay (1942) was cited by Brown (1944) as being successful in separating two hormones originating in the central nervous system.

Summary

1. Chromatophorotropic hormones in three species of Sesarma, *viz., S. intermedia, S. haematocheir* and *S. dehaani,* are differentiated into two broad categories of S and N hormones.

2. S hormone is responsible for pigment concentration in the red and the vermilion chromatophores of juvenile forms of *S. haematocheir,* and also for pigment concentration in the red and the yellow cells of *Paratya compressa,* while N hormone is responsible for pigment concentration in the black chromatophores and pigment

dispersion in the white ones of *S. haematocheir*, juv., and also for pigment concentration in the white cells of Paratya.

3. S hormone is distributed not only in the sinus gland but in all the principal nervous organs in both the adults and the juvenile forms of Sesarma. Occurrence of S hormone in the nervous tissues is independent of the activity of the sinus gland, suggesting the presence of a certain endocrine organization in the nervous system of the crabs.

4. N hormone is restricted to the brain and the Medulla terminalis in the adults, being widely distributed in the brain, the Medulla terminalis, the thoracic and the commissural ganglia in the juvenile forms. Production of the hormone in these ganglionic tissues is also unaffected by the activity of the sinus gland. Stimulation of the optic tract is responsible for secretion of N hormone.

5. Antagonism between S and N hormones could not be detected through the assay with the chromatophores of *S. haematocheir*, juv.

6. The discussion is especially concerned with recent information on the sources and activities of chromatophorotropic hormones originating in the nervous tissues in other species of crustaceans.

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A SEROLOGICAL ANALYSIS OF THE SYSTEMATIC RELATIONSHIP OF THE BRACHYURAN CRAB GERYON QUINQUEDENS

CHARLES A. LEONE

Department of Zoology, University of Kansas, Lawrence, Kansas

The taxonomic relationship of the brachyuran crab *Geryon quinquedens* is open to question. Rathbun (1918) places the species in a sub-family, the Carcinoplacinae, of the family Goneplacidae. Bouvier (1940), on the other hand, lists Geryon as a genus in the family Xanthidae. Dr. Waldo Schmitt, U. S. National Museum, has indicated (personal communication to Boyden in 1935) that morphological studies do not yield a precise placement of this species in the families of the Brachyura.

Boyden (1943) conducted the first serological tests with the serum of *Geryon quinquedens*. His tests, involving among others an anti-Geryon and an anti-Menippe serum, showed reciprocal reactions which indicated as high a degree of relationship between Geryon and Menippe, the latter a member of the family Xanthidae, as either one of these showed with other brachyuran Crustacea.

This paper presents additional serological data on the systematic position of Geryon as revealed through the precipitin reaction.

Materials and Methods

Through the facilties of the Serological Museum of Rutgers University, New Brunswick, New Jersey, it has been possible to assemble serum samples of species of Crustacea which are widely scattered geographically.[1] A list of the species whose sera were used in this report to make a serological analysis of the taxonomic position of Geryon is given in Table I.

Table 1

Table showing the species whose sera were compared by serological means in this paper

Species	Source
Geryon quinquedens Smith	Tortugas, Florida, U. S. A.
Menippe mercenaria (Say)	Unknown
Panopeus herbstii Milne Edwards	Unknown
Eriphia spinifrons (Herbst)	Naples, Italy
Callinectes sapidus Rathbun	Delaware Bay, New Jersey, U. S. A.
Portunus puber Linnaeus	Dinard, France
Cancer pagurus Linnaeus	Dinard, France
Pachygrapsus marmoratus Fabricius	Naples, Italy
Ocypode albicans Bosc	Tortugas, Florida, U. S. A.
Maia squinado Rondalet (Herbst)	Plymouth, England

[1] The author in his capacity as an officer of the Serological Museum in the summer of 1948 collected samples of sera from European Crustacea at marine biological stations in France and Italy. Other sera indicated in Table I were either collected personally by the author or provided from the stocks of the Serological Museum.

The methods for preparing rabbit antisera against the Crustacea hemocyanins and the technique for performing turbidimetric titrations of these antisera with antigens have been described in detail elsewhere (Boyden, 1942; Boyden and De Falco, 1943; Leone, 1949). In brief, the antisera were prepared in rabbits which received intravenously one or two series of four doubling injections of antigen. Serological comparisons were made by measuring with a Libby photronreflectometer (1938) the precipitates which developed when antigens and antibodies were mixed in varying proportions. To each dilution of a doubling dilution series of antigen was added a constant amount of antiserum. The entire reaction range from antigen excess to antibody excess was covered. The antigen-antibody mixtures were incubated for twenty minutes at 38° C. The precipitates which formed in the mixtures were then read in the turbidimeter. The areas of the resulting curves were used as a statistical index of the amount of reactivity between the particular antigen and antiserum being tested.

Experimental Results

The serological data are summarized in Table II.

Table II

Table showing serological relationships among brachyuran Crustacea. Values are given in percentages. Comparative values are read in vertical columns. Family names are given under each scientific name. The homologous test is identified as the 100 per cent reaction. Heterologous antigens react with the antisera in proportion to their degree of serological correspondence to the homologous antigen. Relationship values within 5 per cent of one another are considered to be of the same order of magnitude.

Antigens	Antisera								
	Geryon quinquedens	*Menippe mercenaria* (Xanthidae)	*Eriphia spinifrons* (Xanthidae)	*Callinectes sapidus* (Portunidae)	*Portunus puber* (Portunidae)	*Cancer pagurus* (Cancridae)	*Pachygrapsus marmoratus* (Grapsidae)	*Ocypode albicans* (Ocypodidae)	*Maia squinado* (Majidae)
Geryon quinquedens	100	35	28	59	37	45	64	14	14
Menippe mercenaria (Xanthidae)	39	100	28	54	35		64	34	13
Eriphia spinifrons (Xanthidae)	37	35	100	51	37	46	65	32	13
Panopeus herbstii (Xanthidae)	40	36	26	51	37		65	39	14
Callinectes sapidus (Portunidae)	36	36	23	100	37	48	54	30	21
Portunus puber (Portunidae)	34	35	21	62	100	51	55	30	20
Cancer pagurus (Cancridae)	26	27	18	40	25	100	68	33	16
Pachygrapsus marmoratus (Grapsidae)	16	15	15	25	20	40	100	35	16
Ocypode albicans (Ocypodidae)	15	12	10	21	25	35	64	100	13
Maia squinado (Majidae)	5	12	10	21	25	22	25	5	100

The data obtained with the anti-*Geryon quinquedens* rabbit serum reveal that:

(a) Of all the heterologous antigens, those of the Xanthidae and Portunidae react most. There is no significant difference in the reactivity of the species of these two families with the anti-Geryon serum.
(b) The serum of the cancrid crab reacted only slightly less than the sera of Portunidae and Xanthidae.
(c) Sera of the Grapsidae and Ocypodidae react with the anti-Geryon serum equally but still less than the above families.
(d) Of all the families tested, the representative of the Majidae gave the least amount of reactivity with the anti-Geryon serum.

The results obtained using the anti-*Menippe mercenaria* rabbit serum and the anti-*Eriphia spinifrons* rabbit serum were virtually the same for the heterologous antigens as the results obtained with the anti-Geryon rabbit serum. The serum of Geryon, moreover, reacted as strongly with these two antisera as did any other heterologous antigen tested. In view of this fact, and inasmuch as Menippe, Eriphia and Panopeus are all members of the family Xanthidae, the serological affinities of Geryon could place it in, or close to, this family.

The extent of the reaction of the species of the family Xanthidae, including Geryon, with antisera made against the sera of species of the Portunidae would indicate a close relationship between the two families. In fact, the heterologous Portunidae antigen did not react to any greater extent with an anti-Portunidae serum than did the sera of the Xanthidae. The antigen next most reactive with the anti-*Callinectes sapidus* rabbit serum was from the family Cancridae. These were followed by sera of the Ocypodidae, Grapsidae and Majidae, all equally reactive with the anti-Portunidae serum. The anti-*Portunus puber* rabbit serum was less discriminating than the anti-*Callinectes sapidus* rabbit serum. The former antiserum arranged the antigens which were tested into two principal groups, the one containing the Portunidae and Xanthidae, the other containing the Cancridae, Grapsidae, Ocypodidae and the Majidae. The latter anti-portunid serum set apart the other portunid crab from the Xanthidae, and also separated the Cancridae from the other families tested. It is interesting to note in connection with this antiserum that the proteins of Geryon reacted as much as the proteins of the heterologous portunid crab. This is unusual in view of the discriminating capacity of the antiserum. On the basis of this serological comparison, Geryon could be placed in the Portunidae rather than in the Xanthidae.

The anti-*Cancer pagurus* rabbit serum reacted most strongly with antigens from species in the families Portunidae and Xanthidae. Geryon's serum reacted as much with this antiserum as did the xanthid serum. Sera from species representing the families Grapsidae and Ocypodidae reacted somewhat less than those of the portunid and xanthid crabs. The antigens of *Maia squinado* reacted least of all with the anti-*Cancer pagurus* rabbit serum.

The anti-*Pachygrapsus marmoratus* serum grouped as equally reactive the sera of the species of the Cancridae, Xanthidae, and Ocypodidae. Sera of species from the Portunidae reacted slightly less, and the serum from the family Majidae the least.

Except for the sera of Maia and Geryon, which reacted less than the others, all the heterologous antigens reacted to approximately the same extent with the anti-*Ocypode albicans* rabbit serum. The writer can offer no explanation why the serum of Geryon should be distinguished in this manner from the sera of the xanthid and portunid crabs.

The anti-*Maia squinado* rabbit serum was unable to distinguish preferentially any of the heterologous antigens.

Discussion

Bouvier's classification (1940) of *Geryon quinquedens* is more generally confirmed by serological tests in this report than is the classification of Borradaile (1907) as copied and adapted by Rathbun (1918, 1937). Geryon serologically appears to be more like species in the families Xanthidae and Portunidae than like the species of any other of the families tested. Unfortunately, at the present writing, there is no true goneplacid serum available with which to compare the serum of Geryon or the sera of xanthid and portunid crabs. The relationship of Geryon to the Goneplacidae cannot be conclusively settled until these comparisons are made. The unlikelihood of obtaining the sera of the Goneplacidae, which would make the critical serological analysis possible, has prompted me to submit such data as are available. It is difficult to believe, however, that Geryon could exhibit more serological correspondence to the sera of the Goneplacidae than it has with those of the Xanthidae and Portunidae unless the sera of the Goneplacidae have an amount of serological correspondence to the Xanthidae and the Portunidae similar to that exhibited by Geryon.

The position of the family Cancridae as revealed through the serum of *Cancer pagurus* is interesting. When analyzed with the more discriminating antisera made against the sera of species from the tribe Brachyrhyncha such as the anti-Menippe and the anti-Callinectes sera, the antigens of Cancer are separated from those of species in Bouvier's tribe Corystoidea. Similarly, the anti-*Cancer pagurus* rabbit serum indicated that the xanthid and portunid antigens were more similar to those of Cancer than were the antigens of the grapsid and ocypodid crabs. In contrast to this the less discriminating anti-xanthid and anti-portunid rabbit sera, namely anti-Eriphia serum and anti-Portunus serum, classified the Cancer antigens as being of about the same order of reactivity as the sera of the grapsid and ocypodid crabs. Antisera against the proteins of *Pachygrapsus marmoratus* and *Ocypode albicans* did not give any greater reaction with the serum of Cancer than they did with the sera from species in the other families. The serological data indicates then that with appropriate antisera the proteins of *Cancer pagurus* can be shown to be more similar to the xanthid and portunid crabs than to the grapsid and ocypodid crabs.

Whether or not Bouvier was justified in placing Cancer in the tribe Corystoidea instead of the tribe Brachyrhyncha is a matter of opinion. The criteria which are used to establish different systematic categories are often debatable. In the case of serological data, homologous proteins of two species from different yet closely related tribes might exhibit greater serological correspondence than the homologous proteins of two widely separated species within a single tribe. This is not inconsistent with good systematics.

The anti-Grapsidae and anti-Ocypodidae rabbit sera did not give a greater reaction with the antigens of Geryon over the other heterologous antigens tested. The family Goneplacidae was classified by Bouvier with the Grapsidae and Ocypodidae in the group Catometopa. If this classification is correct, then Geryon is more correctly placed as a genus in the family Xanthidae or in the family Portunidae. However, as indicated above, exact serological relationship of Geryon to the Goneplacidae still remains to be determined.

Summary

1. Serological tests have revealed that the serum of the brachyuran crab *Geryon quinquedens* has a greater degree of correspondence to the sera of species in the families Xanthidae and Portunidae than to the sera of species in the families Cancridae, Ocypodidae, Grapsidae and Majidae.

2. Of the species which were tested serologically, those in the families Xanthidae and Portunidae have the closest relationship. The family Cancridae is closer to the Xanthidae and Portunidae than any other of the families tested.

3. The families Grapsidae, Ocypodidae, and Majidae are quite distinct from one another, and from the other families tested. The family Majidae is serologically least like any of the families tested.

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THE ACTIVITY OF THE CONTRACTILE VACUOLE IN A SUCTORIAN (TOKOPHRYA INFUSIONUM) [1]

MARIA A. RUDZINSKA AND ROBERT CHAMBERS

Department of Biology, Washington Square College of Arts and Science, New York University, and the Marine Biological Laboratory, Woods Hole, Massachusetts

This paper deals with a study of the behavior of the contractile vacuole during several functional phases in the life of *Tokophrya infusionum,* a protozoon of the Class Suctoria.

The Tokophrya has proved to be exceptionally favorable: (a) The sedentary habit of the adult facilitates observation on the contractile vacuole during all periods of its activity. (b) The food supply can be regulated since the organism feeds only on living ciliates. (c) The contents of the prey are incorporated by being sucked out through the predatory tentacles of the host. This eliminates the complication of having water introduced, a usual accompaniment of the formation of food vacuoles in other protozoa. (d) During reproduction a parent organism persists, since Tokophrya reproduces by the formation of a succession of "embryos" in temporary brood pouches.

General Description of Tokophrya

Tokophrya, a fresh water, sessile suctorian, possesses a spheroidal or pyriform body with an average diameter of 17 to 50 micra. Two bundles of straight, motionless, sucking tentacles (5–20 in each bundle) extend, one from each side of the body (Fig. 1A). The contractile vacuole, of which there may be two, rarely three, has an average maximum diameter of 10 micra. Feeding starts as soon as the prey, generally consisting of small living ciliates, becomes attached to one or several tips of the extended tentacles. One Tokophrya may feed simultaneously on ten or more ciliates, each of which may be several times the size of the Tokophrya. In a starved Tokophrya the body is translucent and the macronucleus is then clearly visible. During feeding, the body becomes opaque and filled with numerous dark food vacuoles. With time these vacuoles become increasingly translucent and finally disappear within 24–48 hours. The capture of the swimming prey depends upon its chance contact with the tentacles of the Tokophrya. Shortly after being caught, the prey becomes motionless and, a few seconds later, granules of cytoplasm from the prey can be seen to flow through the hollow tentacles into the host. Within 5 to 10 minutes the prey progressively shrinks in size and, after 25 minutes or more, only a spherical remnant is left, which becomes detached and disintegrates. It is of interest to note that the contractile vacuole of the prey continues to pulsate until just before the prey is reduced to the discarded small remnant.

Tokophrya reproduces by endogenous budding, the bud being produced within a temporary vacuole, the so-called "brood pouch." A well-fed individual is able

[1] This work was supported through funds from the National Cancer Institute of the U. S. Public Health Service.

to produce as many as twelve embryos successively during 24 hours. The first indication of budding is the formation of the brood pouch which appears as a large triangular vacuole at the apical region of the body. After a few minutes the vacuole becomes crescentic as it progressively encircles one of the two mitotically produced

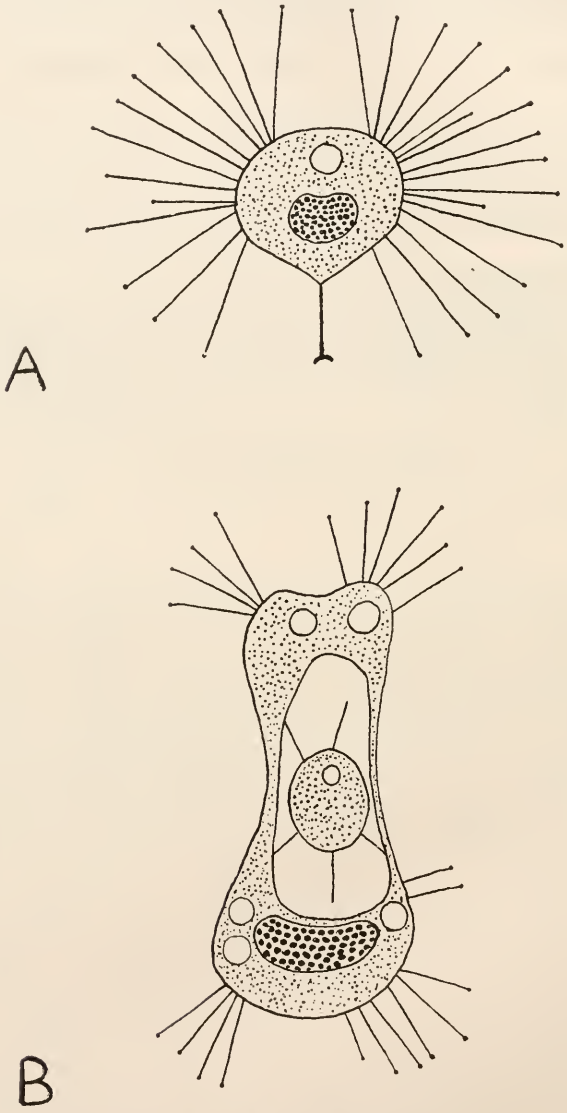

FIGURE 1. A. An adult *Tokophrya infusionum* with two bunches of tentacles, a stalk with an attachment disc, one contractile vacuole and a macronucleus. B. An abnormal case of a *Tokophrya infusionum* in which a young adult persists in the brood pouch. There are five bunches of short tentacles and five contractile vacuoles. The enclosed young Tokophrya has one contractile vacuole and five tentacles, the tips of three of which are in contact with the wall of the brood pouch.

micronuclei, together with some cytoplasm and a small portion of the macronucleus. The embryo, thus constituted, is pinched off, becomes ciliated and rotates within the brood pouch for about 10 to 20 minutes before it is ejected to the exterior. The whole process, from the time of the formation of the brood pouch until the liberation of the embryo, lasts for a little more than an hour. The liberated embryo swims for several minutes to several hours after which it becomes attached, loses its cilia and is transformed in the next few minutes into a mature form. The development and subsequent ejection of the embryo in Suctoria has been fully described by Collin (1911, 1912), Penard (1917–23), Pestel (1931), Noble (1932).

Material and Methods

The material for this study was obtained in the spring of 1948 through the courtesy of Dr. Daniel Lilly, St. John's College, Brooklyn, New York, from a pond in the vicinity of Laurelton, New York. From one of several mass cultures, 20 pure line cultures of *Tokophrya infusionum* were started. These were maintained in standing drops on microscopic slides bounded by walls of thick vaseline or a mixture of paraffin and beeswax. All the cultures were kept in large moist chambers and transfers were made weekly.

Bacteria-free cultures were obtained by transferring a free swimming embryo through several washes of autoclaved Great Bear Spring water. The ensuing progeny were maintained throughout in autoclaved water. The food supply consisted of bacteria-free cultures of *Tetrahymena geleii*. These were grown in a proteose-peptone medium. The cultures of Tokophrya were fed with Tetrahymena three times a week and were transferred to fresh media every three to four weeks.

The contractile vacuole always undergoes complete contraction. Its pulsation rate was recorded by means of a stop watch and its diameter was measured when at a maximum just prior to its periodic expulsion. These measurements were used to calculate the amount of fluid expelled over a given period of time. All studies were made at room temperature.

Experimental

The contractile vacuole during feeding

Fifty individual Tokophrya, selected from pure cultures, which had been maintained without food for 12 to 24 hours, were mounted singly in hanging drops. Into these drops were introduced one, two or three Tetrahymena as food. It was found that in every case the pulsation rate increased suddenly after attachment of the prey, remained high during feeding, and fell abruptly at the termination of the feeding. On the other hand, the maximum diameter was found to vary in different individuals. Out of the 50 examined, it was larger in two, remained the same in 24 and was smaller in 24, during than before feeding. No significant differences were obtained as to pulsation rate or diameter, irrespective of whether the individuals were fed simultaneously on two, three or four Tetrahymena.

Table I presents the values obtained in one individual which was fed with one Tetrahymena and in which the maximum diameter of the contractile vacuole was less during than before feeding. The table presents in four columns (1) the intervals, in seconds, between six successive pulsations, (2) the average maximum diameters, in micra, of the contractile vacuole, (3) values calculated from the average

TABLE I

Activity of the contractile vacuole of a Tokophrya before, during and after feeding

	Intervals in seconds between six successive systoles					Av. maximum diam. of cont. vacuole	Av. amt. fluid expelled per second	Calculated number of systoles per hour
Before feeding	175″	125″	171″	185″	180″	10.4 μ	3.52 μ^3	22
During feeding	22″	23″	24″	23″	21″	9.7 μ	21.15 μ^3	159
Immediately after feeding	45″	45″	47″	45″	46″	9.7 μ	10.48 μ^3	79

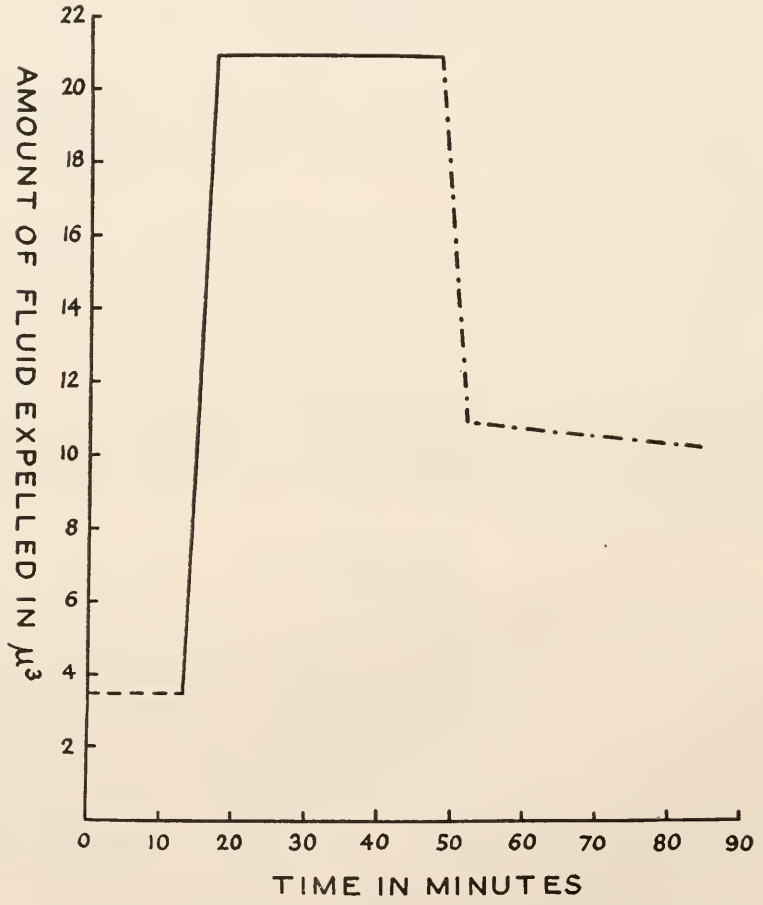

FIGURE 2. The curve represents the amount of fluid expelled by the contractile vacuole before, during and after feeding. The abscissae give the time in minutes; the ordinates, the amount of fluid in μ^3 expelled calculated for 1 sec. Data from Table I.

——————— Before feeding
——————— During feeding
—·—·—·—·— After feeding

amount of fluid expelled in 1 second, and (4) the average number of systoles in one hour. From these figures it can be seen that the amount of fluid expelled by the vacuole averaged six times more during than before feeding. The amount of fluid expelled immediately after feeding was still three times as great as before feeding.

The characteristic features in these experiments are the sudden acceleration of the pulsation rate when feeding started, the increased amount of fluid expelled during and a gradual deceleration of the rate after feeding.

Figure 2 presents graphically this phenomenon. To be noted is the steepness of the upward slope representing the increase of fluid expelled when feeding has begun. The following horizontal part of the curve shows the constancy in the activity of the contractile vacuole during feeding. The subsequent rapid fall of the curve represents the decrease when feeding had ceased. Following this the curve drops gradually and, 35 minutes after feeding, is still far from the low level before feeding. Measurements were continued up to several hours after feeding at which time the level was still appreciably high.

Table II presents a striking fact that the increase in volume of the Tokophrya during feeding is appreciably less than might be expected on the basis of the volume of the food ingested. One Tetrahymena is about two to ten times larger than one

TABLE II

Volumes of three spherical Tokophrya before and after feeding. The three individuals were fed with one, two and three Tetrahymena respectively

	Fed on one Tetrahymena	Fed on two Tetrahymena	Fed on three Tetrahymena
Volume before feeding	12,000 μ^3	9,000 μ^3	11,000 μ^3
Volume after feeding	22,000 μ^3	25,000 μ^3	38,000 μ^3
Increase in volume	10,000 μ^3	16,000 μ^3	27,000 μ^3

Tokophrya. Provided the entire mass of the prey is incorporated, the Tokophrya, after having ingested one Tetrahymena, should increase in size by 200 to 1000 per cent instead of only about 80 per cent as actually occurs. This appears in the table which gives the volumes in cubic micra of each of three spherical Tokophrya before and after being fed with one, two and three Tetrahymena respectively. The table shows that in all three cases the increase in volume was definitely less than it should be when one considers the volume of the prey incorporated. The Tetrahymena is irregularly cucumber-shaped and its volume had to be estimated by considering it as an ellipsoid with long and cross axes of 60 and 24 micra respectively. The calculation gave a figure of 47,000 micra.

Calculations were also made of the volume of material actually incorporated by measuring (1) the time taken for the ingestion to take place, (2) the rate of movement of the granules through the tentacles from the prey to the host, and (3) the number, diameter and length of the predatory tentacles. The value obtained from these calculations accorded closely with that of the actual volume of the prey.

The difference between the expected and the actual increase in the volume of the host, as shown in Table II, can be accounted for by the amount of fluid expelled from the host by its contractile vacuole. This difference approximates the value

calculated from the estimated volume of the ingested Tetrahymena (44,000 μ^3) and the amount of excess fluid expelled by the contractile vacuole of the Tokophrya during feeding (roughly 40,000 μ^3) minus the amount of fluid (6,300 μ^3) expelled before feeding. The figure, 10,300 μ^3, which is 44,000 − (40,000 − 6,300), closely approximates the value of 10,000 μ^3, shown in Table II, of the Tokophrya fed with one Tetrahymena.

The contractile vacuole during reproduction

Experiments were performed to determine the effect of reproduction on the activity of the contractile vacuole. Unfed Tokophrya were used. Some were young adults kept without food since metamorphosis, others were adult Tokophrya which had been maintained without food for at least 12 hours.

It was found that the process of reproduction greatly accelerated the pulsation rate of the contractile vacuole and the amount of fluid expelled. This acceleration was progressive, starting from the time the brood pouch could be seen beginning to form, and reaching a maximum when the ciliated embryo was being completed as an isolated individual within the brood pouch. From that time on, the activity of the contractile vacuole of the mother decelerated, fell greatly by the time the embryo was expelled and still more so when the expulsion was complete. This last value was below the level determined at the beginning of the experiment and is to be interpreted as that of the individual before the reproductive process had started. At the time when the observations were made, the brood pouch had already begun to form and, therefore, the activity of the contractile vacuole presumably was already on the increase.

TABLE III

Activity of contractile vacuole in a Tokophrya during reproduction

Reproductive stages	Time in minutes	Intervals in seconds betw. six successive systoles					Av. maximum diam. of contr. vac. in μ	Av. amt. fluid expelled, μ^3/1 sec.	Calculated number of systoles per hour
Brood pouch initiated	0	86	87	90	91	82	11.7	9.66	41
Cilia of embryo in action	15	82	80	74	67	67	11.7	11.50	49
Contractile vacuoles of embryo in action	25	44	40	37	35	35	10.4	15.50	95
Embryo being completed and starting to rotate	30	27	25	25	27	29	10.4	21.82	133
Embryo being expelled	67	55	59	60	60	60	9.8	8.36	61
Expulsion completed	75	80	90	107	120	120	9.8	4.79	35

Table III presents figures for the intervals, in seconds, between the pulsations and the maximum diameter of the contractile vacuole during successive stages of the reproductive process, *viz.*, (a) the initiation of formation of the brood pouch, (b) the beginning of the ciliary activity of the forming embryo, (c) the time when the contractile vacuoles of the embryo came into action, (d) the separation of the embryo into an isolated individual, (e) the beginning and (f) the completed expulsion of the embryo.

Figure 3 represents graphically this phenomenon. The upward slope of the curve represents the increasing vacuolar activity from the moment the brood pouch

started to be formed until the peak is reached when the embryo was fully developed. The subsequent fall represents the decrease of the vacuolar activity after the completed growth of the embryo.

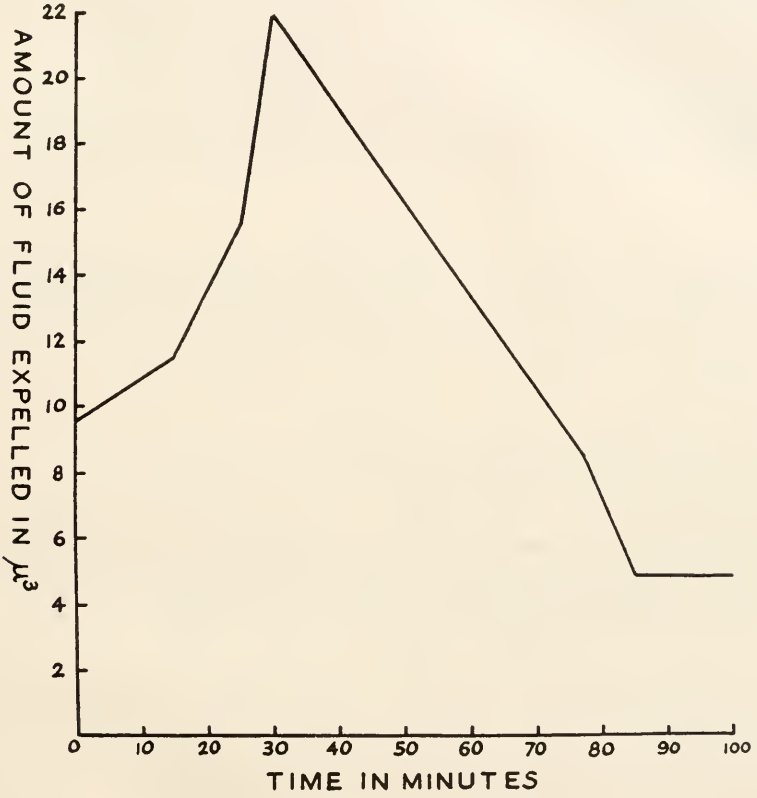

FIGURE 3. Successive changes in the amount of fluid expelled during the reproductive period. The abscissae give the time in minutes; the ordinates, the amount of fluid in μ^3 calculated for 1 sec. Data from Table III.

The contractile vacuole in individuals with abnormal reproduction in which the embryo underwent metamorphosis but persisted as an adult within the brood pouch

The contractile vacuole of these abnormal Tokophrya (Rudzinska, in press) was studied in order to ascertain whether the presence of the young adult, completely enclosed within the mother and with its tentacles attached to the wall of the brood pouch, had any effect on the activity of the contractile vacuole of the mother. In the six abnormal Tokophrya studied, it was found that the mother developed large, supernumerary contractile vacuoles which pulsated at a faster rate than normal. This is shown in Table IV, which is to be compared with Table I.

Of these abnormal individuals, four had been kept unfed. The remaining two were fed and in these the effect of feeding was evident by the still greater acceleration of the activity of the contractile vacuole.

TABLE IV

Activity of three contractile vacuoles in a Tokophrya with a young adult persisting within the brood pouch

	Intervals in seconds between six successive systoles														
	Vacuole 1					Vacuole 2					Vacuole 3				
Before feeding	50	48	45	45	48	55	52	50	53	50	60	55	55	58	55
After feeding	13	13	10	10	12	10	12	10	12	12	13	10	10	12	10

DISCUSSION

In a review concerning the function of the contractile vacuole in protozoa, Weatherby (1941) refers to many investigators whose observations, he states (p. 430), "strongly suggest that the vacuole operates to prevent excessive dilution of the cytoplasm, or to regulate osmotic pressure within the cell. . . ." However, he concludes (p. 430): "In spite of the quite extensive literature dealing with the question, one is obliged to admit that virtually nothing has been proved beyond question concerning the function or functions of contractile vacuoles."

The favorable feature of Tokophrya is that the study of the activity of the contractile vacuole can be closely related to various phases of metabolic activity. These include feeding, reproduction and an unusual case in which the embryo underwent metamorphosis and persisted as an adult within the body of the mother.

The effect of feeding in increasing the pulsatory rate of the contractile vacuole has already been described by other investigators. Pestel (1931) observed this in the suctorian, *Dendrocometes paradoxus* Stein. Andrejewa (1931) found that in *Paramecium caudatum* the greater the number of food vacuoles, the faster is the pulsation rate of the contractile vacuole. Some investigators (Kitching, 1938) claim that the increased activity of the contractile vacuole with feeding is to be accounted for by the amount of water incorporated from the exterior during the formation of the food vacuole. However, such an explanation is excluded in Tokophrya since the only material incorporated is the contents of the prey. If the fluid expelled is only that taken in unchanged from the Tetrahymena it would be expected that when feeding is completed the pulsation rate should return soon to the previous rate. However, this does not occur. The contraction rate falls only gradually and still is appreciably high for as long as several hours after feeding. This indicates the possibility of another factor responsible for the persisting high rate of output, namely, an increase in the metabolic activity of the Tokophrya. Such a relation is supported by the findings of Shumway (1917), Riddle and Torrey (1922) and Flather (1919) that thyroxin, adrenalin, pituitary and pineal gland extract accelerate the pulsation rate of the contractile vacuole. The correlation between the activity of the contractile vacuole and increased metabolism is also suggested in those cases of Tokophrya in which the young adult persisted virtually as a parasite within the brood pouch of the mother. The increased number and rapid contraction rates of the contractile vacuoles in these mothers may be accounted for by the metabolic activity of the imprisoned, parasitic offspring. The contractile vacuoles of the mother evidently are doing double duty for herself and for her enclosed adult offspring.

The increased rate of pulsation during reproduction has also been described in the literature. Unger (1925, 1926) studied the activity of the contractile vacuole of *Paramecium caudatum, P. aurelia* and *P. calkinsi* in connection with their division rate and during endomixis. He found a relation between a reduction in the pulsatile rate of the contractile vacuole and a reduction in the rate of division and of endomixis. Dimitrowa (1928) observed that at the beginning of mitosis in *Paramecium caudatum* two additional contractile vacuoles appear and claimed this to indicate increased excretory activity. Pestel (1931) observed that the first sign for endogenous budding in *Dendrocometes paradoxus,* a suctorian, is the formation of a small contractile vacuole in that part of the cytoplasm which is destined to be the embryo.

A feature of this paper is that it has been possible to show in the same species, Tokophrya, three kinds of functional activity to each of which the contractile vacuole responds specifically. These are a response (1) to feeding, (2) to the process of reproduction, and (3) to the parasitic existence of the offspring enclosed within the body of the host.

Summary

1. Evidence is presented for the existence of a close relation between the behavior of the contractile vacuole and several phases of the functional activity of *Tokophrya infusionum.*

2. When the process of feeding starts the pulsation rate of the contractile vacuole rises suddenly, remains high during feeding and falls gradually on cessation of the feeding process. It is significant that the pulsation rate remains comparatively high for several hours after the termination of the feeding.

3. During reproduction the pulsation rate of the contractile vacuole increases as soon as the endogenous budding starts, rises and remains high until the ciliated embryo has become separated within the brood pouch. This moment is the high peak of the vacuolar activity. The pulsation rate subsequently begins to decrease and falls greatly upon expulsion of the embryo. After expulsion, which lasts several minutes, the pulsation rate of the contractile vacuole drops still lower.

4. Unusually accelerated activity of the contractile vacuole was noted in those cases in which metamorphosis of the embryo occurred within the parent and the resulting young adult persisted with the tips of its tentacles attached to the wall of brood pouch.

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THE MARINE SAND-DWELLING CILIATES OF CAPE COD

E. FAURÉ-FREMIET

Collège de France, Paris, and Marine Biological Laboratory, Woods Hole, Mass.

Marine Sands and Psammophilous Ciliates

It is well known that the marine sands between the tidemarks, or deeper along the seashore, constitute some quite peculiar and interesting biotopes. With regard to their fauna, one can distinguish the sand-boring animals of large or small size and, on the other hand, the very minute ones which live in the intergranular spaces of the sand and constitute an "interstitial" microfauna.

This interstitial microfauna, or mesopsammon, includes, besides numerous copepods, nematodes, gastrotrichs, etc., many protozoa and most particularly numerous ciliates. Kahl (1933, 1935) has recorded more than 80 ciliate species, belonging to about 20 different families, from the sandy bottoms of Kiel and Helgoland.

I found many of these infusorian species and some new ones in the sand of the tidal zone of different beaches in Brittany (Fauré-Fremiet, 1950). This result ties up with some other dispersed data and agrees with a general statement of Calkins (1933) who emphasizes the cosmopolitan distribution of the marine littoral protozoan species. But, from an ecological point of view, it can be noted that the size of the intergranular spaces acts as a selective factor in the distribution of the interstitial ciliates.[1]

The intergranular spaces may be roughly estimated from the dimensions of the solid grains which bound them; a comparison of the granulometric curves plotted for different kinds of sand with the associated interstitial fauna leads one to distinguish in the fauna two different types of ciliates.

If the mean size of the sand grains exceeds 0.4 mm., the interstitial ciliate fauna includes some characteristic sand-living species and some occasionally sand-dwelling infusorians which are much more numerous. The characteristic sand-living species are generally characterized by their small or medium size, their flattened shape, and their jerky motion, with alternately very fast swimming and sudden stopping when in contact with solid surfaces. The occasional sand-dwellers, quite varied in shape and size and usually widespread along the seashore, display a common but more or less accentuated thigmotactic behavior. All these together constitute, in the medium and coarse sands, what I have called the mesoporal infusorian fauna (Fauré-Fremiet, 1950).

If the mean size of the sand grains falls between 0.1 and 0.3 mm., their interspaces shelter some very peculiar species of ciliates, generally thread-like or ribbon-like, often as long as one or two millimeters, always strongly thigmotactic. These

[1] In a previous paper (Fauré-Fremiet, 1950) are discussed some of the physical and chemical conditions which depend on the enormous development of the solid-liquid interfaces of the sand; they probably play an important role in interstitial life of the sand-dwelling ciliates.

characteristic sand-dwelling species constitute what I have called the microporal infusorian fauna.

It must be said, however, that some ciliates of the microporal type can live as well in the coarse as in the fine sand, and these species may be called euryporal.

Finally, if the mean size of the sand grains is below 0.1 mm., or if a coarser sand is so muddy that the intergranular spaces are filled with very minute organic particles, the porosity becomes incompatible with the interstitial life of any species of ciliates.

Numerous observations made on different beaches and sandy flats of the Cape Cod region during the months of June and July, 1950, offer new evidence of the cosmopolitan distribution of many psammophilous ciliate species, and of the general significance of the two types of infusorian interstitial fauna.

Technique

The superficial layers of wet sand exposed at low tide were collected with a spoon, to a depth of 2 to 10 millimeters, and put in some convenient bottle or carton, with just a little sea water. At the laboratory, the samples were transferred to large fingerbowls, covered with sea water and allowed to stand overnight.

In order to observe the ciliates, a small quantity of sand, taken from one sample with a spatula or a pipette, was put in a Petri dish and gently agitated with sea water to which a few drops of a 12 per cent $MgCl_2$ solution were added.[2]

Then the sand was gathered at one side of the dish and the water examined under a low-power binocular microscope. The psammophilous ciliates, now swimming or gliding on the glass bottom of the dish, are easily picked up with a capillary pipette and transferred to a slide for a more careful examination, or placed in small covered dishes with normal sea water and sand grains for later examination.

The granulometric analyses of three different types of sand were made at the Woods Hole Oceanographic Institution. The corresponding curves were plotted for the very fine sand of the Barnstable Harbor flats, and for the two coarse sands from Tarpaulin Cove and Nobska beaches (Fig. 1).

A rough micrometric estimation of the mean diameter of the grains was quite sufficient for the comparative study of many other samples.

Mesoporal Infusorian Fauna

The sands of almost all the beaches around Woods Hole shelter ciliate species of the mesoporal type.

The samples examined were collected in several places on different beaches at Penzance, Woods Hole (M. B. L. beach), Sippiwisset, Quisset, Gansett and Nonamesset along the Buzzards Bay coast, and at Tarpaulin Cove, Nobska, Falmouth, and Great Pond along the Vineyard Sound.

In every case the granulometric measurements show that the size of most of the sand grains lies roughly between 0.3 mm. and 1.0 mm. These sands are quartzous, with very few mica flakes, and contain, with few or no mud particles, a variable amount of organic residues.

[2] See Fauré-Fremiet (1950). Magnesium chloride, which narcotizes many small metazoans rapidly, decreases the thigmotactic adherence of the ciliates, and hence releases them from the sand particles, without a sensible alteration of their motility.

In these somewhat coarse sands, the ciliates are generally neither abundant nor very varied; they belong most often to diatom-ingesting species.

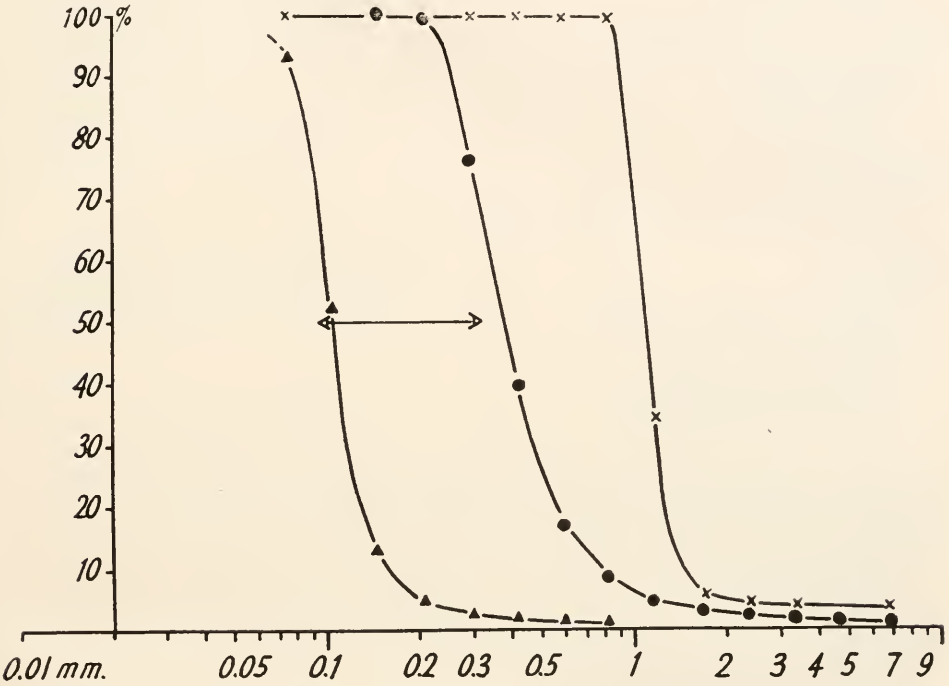

FIGURE 1. Granulometric characteristics of sands collected on three different beaches of Cape Cod. Cumulative curves; abscissa: diameter, in millimeters, of the sand grains (logarithmic scale); ordinate: weight per cent of the sand grains larger than the corresponding diameter. ▲ Barnstable Harbor flat; ● Tarpaulin Cove; × Nobska beach.

The typically mesoporal species observed are the following:

Holotricha Gymnostomata:

Mesodinium pulex, f. *pupula* Kahl
Placus (*Thoracophyra*) *buddenbrocki* Sauerbrey
Remanella margaritifera (?) Kahl

Oligotricha:

Strombidium sauerbreyae Kahl

Hypotricha:

Discocephalus rotatorius Ehrb.

The other species, widely distributed along the seashore, belong occasionally, but not specifically, to the mesoporal fauna; the following are frequently encountered:

Holotricha Gymnostomata:

Lacrymaria sp.
Coleps tesselatus

Holotricha Hymenostomata:

Pleuronema sp.
Frontonia sp.

Heterotricha:

Condylostoma patens (O.F.M.) Stein

Hypotricha:

Epiclintes ambiguus O.F.M.
Euplotes sp.
Diophrys scutum Duj.
Uronychia transfuga O.F.M.
Aspidisca major Madsen

Finally, some sand-living ciliates of euryporal character are often present:

Holotricha Gymnostomata:

Trachelocerca phaenicopterus
Trachelocerca sp.

Hypotricha:

Holosticha fasciola (?) Kahl

It is apparent that these different vegetarian species seem to be living quite independently of one another. Their quantitative distribution appears quite variable and unequal, not only from one beach to another but also along the same beach, between two areas only a few meters distant and showing the same physical appearance. Sometimes a local population of one of these species may be fairly abundant, probably in connection with the local growth of some species of diatom or bacteria utilized as food. This local growth is conditioned itself, perhaps, by some slight local difference in the intensity of light or the tidal agitation.

Microporal Infusorian Fauna

Some of the special conditions needed by the ciliates of the microporal type are found in the sand of the Barnstable Harbor flats, on the south coast of Cape Cod Bay. There the sand is very fine, slightly muddy, closely packed, and inhabited by many burrowing invertebrates, such as clams and worms.

The ciliates seem to be confined to the superficial layer of clear gray sand, sometimes reddish spotted by purple bacteria, and covering a deeper black sand rich in organic decomposition products.

The size of the sand grains varies from 0.05 mm. to 0.2 mm. In many samples collected on the flats exposed at low tide, there was found a more or less abundant and varied association of the following characteristic microporal species:

Holotricha Gymnostomata:

Helicoprorodon (*Chaenea*) *gigas* Kahl
Centrophorella fasciolata Sauerbrey
Centrophorella lanceolata n. sp.
Geleia simplex n. sp.
Geleia decolor Kahl
Geleia fossata Kahl
Geleia orbis Fauré-Fremiet
Remanella sp.

Heterotricha:

Condylostoma remanei Spiegel, Kahl
Blepharisma clarissimum Anigstein
Blepharisma sp.?

Hypotricha:

Urostrongylum caudatum Kahl

Besides this very specific microporal fauna of ciliates, one observes some of the euryporal species already mentioned, and the very curious *Parablepharisma pellitum* Kahl; this is much more abundant in a different biotope, such as the felting of a dense algal littoral flora in some brackish-water ponds.

It can be noted that on the Barnstable flats, as well as on the Brittany beaches, the different species of Geleia behave like saprobic ciliates; they are fairly abundant where purple bacteria grow. On the other hand, different species of Centrophorella, Remanella and Blepharisma are commonly encountered in the same biotope with Geleia, and they may also be found in clean fine sand and occasionally in the somewhat coarse sand of the open beaches, with some of the characteristic species of the mesoporal infusorian fauna.

Comparative Study of the Ciliate Interstitial Fauna

According to the results above, the mesoporal and the microporal infusorian fauna show respectively the same characteristics in the littoral sands of Cape Cod and in those of Brittany. However, the mean size of the sand grains available for each of the two different interstitial faunas seems to be slightly lower on Cape Cod than on the Brittany coasts; this may be due to the presence in Brittany beaches of numerous mica flakes which assure a more compact packing of the sand grains than the almost pure quartzous particles of the Cape Cod sands. In other words, with equal diameter of sand grains, the interspaces are larger between angular particles than between flat and angular particles mixed together; it is known that the dimensions of these spaces act as an important ecological factor in the distribution of ciliates (Fauré-Fremiet, 1950).

As a matter of fact, among the different Cape Cod sandy shores examined, the typical microporal infusorian fauna was only encountered in sands of the Barnstable Harbor flats. Very fine, slightly muddy, filled with numerous sand-boring animals such as annelids and clams, rich in organic residues and in sulfur bacteria, this sand resembles closely in its microfauna the sand of one beach studied in

Brittany, namely, the beach of Mousterlin, near Concarneau (Fauré-Fremiet, 1950). However, Barnstable Harbor shows some estuarine characteristics, such as some tidal variations of the sea water salinity.

Considering the cosmopolitan character of the marine littoral ciliates, the data gained from study of the Cape Cod sandy shores need some discussion. Many of the ciliates found there can be identified without hesitation, and belong to species already described, and found in the European beaches or sandy grounds. Some others appear very closely related to known species. Finally, some seem to be new species.

The following species in the sands of the Cape Cod region were positively identified with those described in the Brittany coast (Fauré-Fremiet, 1950) and the Bay of Kiel (Kahl, 1933, 1935):

Holotricha Gymnostomata:

Mesodinium pulex f. *pupula* Kahl
Placus (*Thauracophrya*) *buddenbrocki* Sauerbrey
Helicoprorodon (*Chaenea*) *gigas* Kahl (Fauré-Fremiet, 1950)
Centrophorella fasciolata Sauerbrey, Kahl
Coleps tesselatus Kahl

Heterotricha:

Condylostoma patens O.F.M.
Condylostoma remanei Spiegel
Blepharisma clarissimum Anigstein
Parablepharisma pellitum Kahl

Hypotricha:

Epiclintes ambignus O.F.M.
Urostrongylum caudatum Kahl
Discocephalus rotatorius Ehrb.
Diophrys scutum Duj.
Uronychia transfuga O.F.M.

Among these ciliates, *Discocephalus rotatorius* requires special mention; this apparently uncommon infusorian, first described by Ehrenberg from the seashore of the Sinai peninsula, was again observed by Sauerbrey (1928) on the sand grounds of the Bay of Kiel. The form found in the coarse sand of Tarpaulin Cove shows not only the same morphological characteristics, but also the same physiological peculiarity reported by Sauerbrey, namely, the ingestion of small sand particles.

Some species, such as *Condylostoma patens,* show great variability of shape and size, but not of structure; however, this does not make identification difficult. On the other hand, it is hard to decide for some other species whether the differences observed between populations living in different regions are of specific value, or merely define some geographic varieties, or result from poor descriptions in the the literature.

Among the Holotricha, *Geleia orbis,* very abundant in the sand of Barnstable Harbor, seems to be more flattened and more thigmotactic than the Brittany form

(Fauré-Fremiet, 1950), but one does not observe any significant differences between these, either as to size (up to 1.5 mm. in full extension) or structure.

Gelcia decolor Kahl is apparently identical with the Brittany species which I described under this name, but both are slightly smaller than the Kahl species and differ perhaps from his by the presence of a little anterior and preoral pit.

Among the Heterotricha a species of Blepharisma with a beaded macronucleus, very common in Barnstable Harbor, should be considered as *B. clarissimum* forma *arenicola* Kahl; it is smaller than the latter form, however, being 275 to 300 micra as opposed to 450 micra (according to Kahl). It seems to be similar to a form observed by Dragesco (personal communication) in the sand at Roscoff. On the other hand, *B. clarissimum* Anigstein is so well characterized by its cytoplasm (refringent, transparent and hyaline, like that of an Opalina) that the so-called "forma arenicola" must be considered as a distinct species.

Among the Hypotricha, a microporal Holosticha with numerous macronuclear beads is shaped nearly like *H. extensa* Kahl, with two frontal cirri; it is a little larger than Kahl's species (about 300 micra *contra* 140 to 240 micra) and possesses four or five transverse cirri like *H. fasciola* Kahl, as against 6 to 7 as in *H. extensa*. But the latter species is narrower and possesses three frontal cirri at least.

A mesoporal Aspidisca, measuring about 50 to 55 micra, resembles very closely *A. major* Madsen, in its two elongated macronuclear pieces and its five transverse cirri (as opposed to 6 for *A. binucleata* Kahl, and 11 to 12 for *A. polystyla* Stein). However, the Madsen species is larger: 60 to 90 micra.

Three presumably new species need now a more detailed description.

New Species

Centrophorella lanceolata n. sp. (Fig. 2)

The enigmatic genus Centrophorella (see Kahl, 1933, 1935 and Fauré-Fremiet, 1950), first described by Sauerbrey under the name Kentrophoros, probably belongs to the family Amphileptidae, according to Kahl.

The different species are strongly thigmotactic, very fragile and difficult to examine *in vitro*. The use of $MgCl_2$ solution shows that they are much more frequently represented in the microporal interstitial fauna than it was supposed at first. I did not observe in the fine sand of Barnstable Harbor the very large species *C. fistulosa* Fauré-Fremiet of the Brittany coast, but I found, besides *C. fasciolata* Sauerbrey, a new one which I call *C. lanceolata* n. sp. It seems highly probable that it can be found elsewhere, and a form recently observed at Roscoff, in Brittany, by Dragesco (personal communication) is perhaps the same.

Centrophorella lanceolata is a very flattened and ribbon-like ciliate, measuring about 460 to 520 micra in length, 40 micra wide and 5 to 6 micra thick. It tapers at the ends; the anterior part is slender, with a sort of neck preceding the buccal edge, curved on the right side and bearing a row of long cilia. The posterior end is pointed.

The ventral surface is ciliated, and the dorsal one is covered with an accumulation of dark sulfur bacteria, except at a narrow lateral margin at the neck and at the tail.

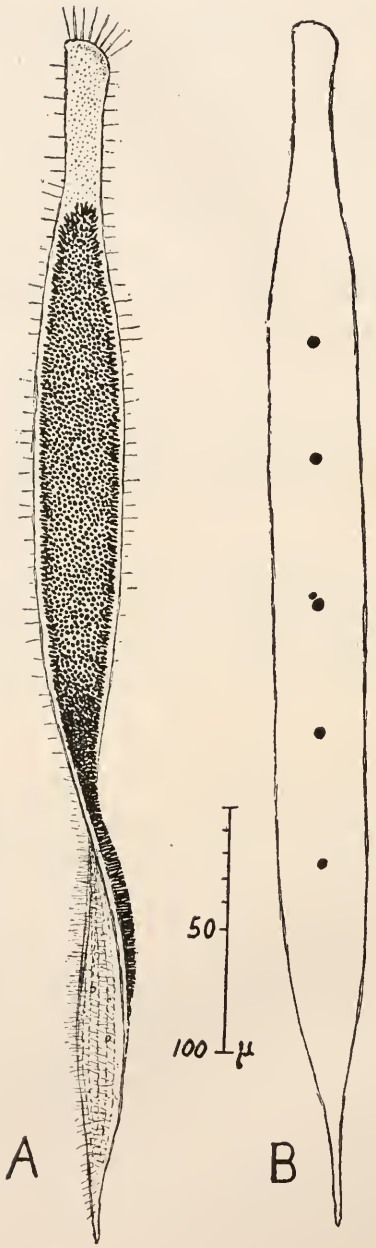

FIGURE 2. *Centrophorella lanceolata* n. sp. A: View of the living infusorian, showing the dorsal brush of sulfur bacteria. B: Outline of the ciliate showing, after staining, the size and the position of five macronuclei and one (probably) micronucleus.

The associated sulfur bacteria, rod-shaped and all of the same length, measure about 10 micra; they are attached to the dorsal surface of the ciliate infusorian body by one end, and arranged in a parallel manner like the bristles of a brush. That is a characteristic of the so-called Caulobacteria—the genus Pasteuria (Metschnikoff), for example. As is the case among others of this group, they divide longitudinally.

The dark appearance of the bacteria of *C. lanceolata* is due to the presence in their cytoplasm of highly refractile sulfur granules; in fact, these bacteria are really colorless, as are those of *C. fistulosa,* whereas those of *C. fasciolata* show a pink color.

It is known that the nuclei of the Centrophorella (Kahl, 1933, 1935; Fauré-Fremiet, 1950) are very small; *C. lanceolata* shows five to six globular macronuclei about 4 micra in diameter, and some smaller micronuclei.

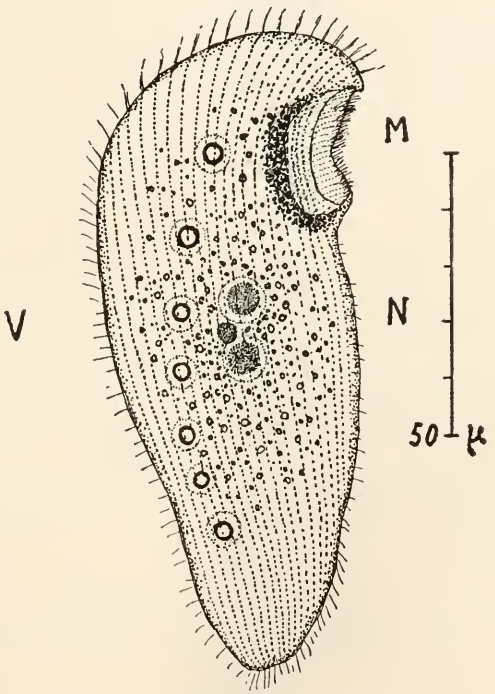

FIGURE 3. *Remanella obtusa* n. sp. View of the ciliated right side. M, mouth; N, double macronucleus with one micronucleus; V, Müller's vesicles with refringent bodies.

The association of the Centrophorella with the Caulobacteria is a perfectly constant character; in each case these bacteria are sulfur bacteria, but they differ in size and color with each species of Centrophorella. It would be interesting to define the nature, specific or adaptively induced, of these differences. On the other hand, it is noteworthy that the bacteria constantly associated with a heterotrichous ciliate, *Parablepharisma pellitum* Kahl, are quite different and do not contain sulfur.

Remanella obtusa n. sp. (Fig. 3)

Whereas the different known species of Remanella belong to the microporal interstitial fauna, this new one was found many times in the relatively coarse sand of Nobska beach.

At first sight, *R. obtusa* recalls some species of Chlamydodon by its shape and its thigmotactic behavior. This ciliate is very flat, yellowish in color, with a brown pigmented area around the mouth. It measures about 110 micra in length and is 40 micra wide. Its anterior edge is broadly rounded.

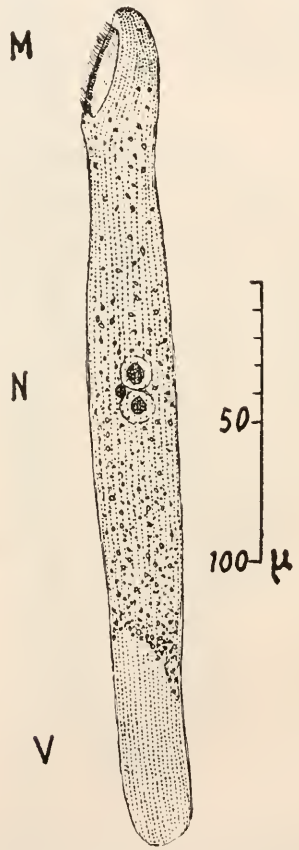

FIGURE 4. *Gelcia simplex* n. sp. M, mouth; N, double macronucleus with one micronucleus; V, large posterior contractile vesicle.

The mouth appears as a lateral semi-circular notch without pharyngeal prolongation. The Müller's vesicles, with a large refringent granule in each one, are 6 to 8 in number. The animal possesses two macronuclei, each containing a central chromatic body, and a single micronucleus.

Remanella obtusa is ciliated only on its flat right side; it is very thigmotactic, and glides rapidly on the surface of the sand grains. It is a diatom feeder.

Geleia simplex n. sp. (Fig. 4)

Geleia simplex, frequently encountered in the fine sand of the Barnstable Harbor flats, differs from the other species of the genus (see Kahl, 1933, 1935; Fauré-Fremiet, 1950) by the lack of an anterior prebuccal lobe, the nearly apical location of the mouth, and the presence of a large posterior vacuole. Its length is about 260 to 300 micra, and its width about 30 micra. Its flexible and slightly contractile body is more or less flattened.

G. simplex possesses two macronuclei with large karyosomes, and one micronucleus.

The mouth is an oval depression extending anteriorly between two body rows of cilia. The buccal ciliation includes a fringe of large cilia on the right side and on the left one a field of fine undulating cilia.

This work was done at the Marine Biological Laboratory under a grant of the Commonwealth Fund, and with the aid of the American Philosophical Society. It gives me great pleasure to acknowledge here the assistance of Mr. Turner, at the Woods Hole Oceanographic Institution, in the exploration of the Barnstable Harbor flats and the measurements of some sand samples. I am grateful to Dr. M. Harnly and Dr. G. Kidder, who read the manuscript.

Summary

1. Two categories of ciliate species dwell in the damp sand of marine beaches; some of these seem to be so closely adapted to interstitial conditions that they do not live outside; some others enter the sand readily and can live in it as well as anywhere else along the seashore, where they are ordinarily widespread.

2. With regard to the size of the interstitial sand spaces, which are roughly proportional to the size of the grains, the sand-dwelling ciliates can be divided into two ecological classes:

In coarse sands, with a mean diameter of the grains above 0.4 mm., the mesoporal infusorian fauna includes a few species exclusively sand-living and many occasional sand-dwelling forms.

In fine sands, with a mean grain diameter of 0.3 to 0.7 mm., the microporal infusorian fauna is composed of true sand-living ciliate species.

In addition, some typical interstitial species, widespread in fine and coarse sands, are called euryporal.

3. Comparison of the ciliate interstitial fauna observed in Brittany and Cape Cod beaches shows that interstitial species are cosmopolitan, and that the distinguishing characteristics of mesoporal and microporal fauna have a generalized ecological significance. On the other hand, it appears that the truly microporal species are endowed with some peculiarities very interesting from the standpoint of cytology, cell-physiology and micro-ecology.

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STUDIES IN THE PHYSIOLOGY OF COMMENSALISM. 2. THE POLYNOID GENERA ARCTONOË AND HALOSYDNA

DEMOREST DAVENPORT AND JOHN F. HICKOK

University of California, Santa Barbara College, and David Starr Jordan Junior High School, Burbank, California

In earlier experiments (Davenport, 1950), it was demonstrated that the specificity of the commensal polynoid worms *Arctonoë fragilis* (Baird) and *A. pulchra* (Johnson), respectively inhabiting the starfish *Evasterias troschelii* Stimpson and the sea-cucumber *Stichopus californicus* (Stimpson), could at least in part be explained by a positive response which these commensals showed to their hosts. On the basis of these tests it was concluded that "certain echinoderm hosts produce a specific diffusible substance that acts as a powerful attractant for their commensals."

Welsh (1930, 1931) had demonstrated that similar agents govern the specificity of certain clam-acarine partnerships. In the course of his work these chemical agents were found to be relatively stable. Boiling for five to ten minutes as well as putrefaction for a week at 37.5° C. had little effect on the material acting on the mites. Beyond this, no further investigation of the nature of the substance was carried out.

To the writers it also seemed of primary importance to determine whether or not the attractants involved in echinoderm-annelid partnerships are stable, as a first step in an investigation of the nature of these substances. In addition, questions had arisen as to the source of the attractants in the host. The necessity for investigation of the factors determining the specificity of further commensal partnerships involving diverse phyla was, as always, present.

In the summer of 1950, experiments designed to attack some of the above problems were conducted at the Oceanographic Laboratories of the University of Washington at Friday Harbor, under grants from the American Philosophical Society and the Society of Sigma Xi. The writers wish to express their appreciation to the Societies and to the Director and staff of the Laboratories for their generosity and assistance.

The Genus Arctonoë

Material

Evasterias and Stichopus with their attendant commensals were collected in the same localities and in the identical manner as described in earlier work (Davenport, 1950). For one series of experiments, however, a large number of Evasterias averaging the size of a silver dollar (3–15 cm.) were collected on a point near the village of Olga on Orcas Island. These immature starfish did not seem to have as

many commensals (5 out of 41) as a comparable population of adults, on a third or more of which one would find worms. At this particular locality one could collect great numbers of immature stars, but extremely few adult ones; it would seem that immature populations are as locally distributed in the Sound inter-tidal as are adult ones.

Methods

The equipment described for earlier experiments (Davenport, 1950) was used, and an additional apparatus was also developed so that two experiments could be conducted simultaneously. The aquaria and the Y tubes of this new apparatus were constructed of lucite, the aquaria being built of one-foot square, ¼" lucite and the Y tubes of ¾" tubing. In order to reduce the possible effects of light, the Y tubes were painted with an opaque black paint along the sides of the stem and arms, leaving a median unpainted area for observation of the worms.

Methods of handling the animals, conducting experiments and recording data were as described previously, except that it soon became clear that in order to demonstrate a positive response, it had neither been necessary to separate the populations of commensals from their hosts for a period of time, nor to keep hosts for a time in non-circulating sea-water before testing so that attractant could accumulate. In control tests it was shown that worms which had just been removed from their hosts would give a series of strong positive runs if trials were commenced from 10 to 15 minutes after a host was placed in the test aquarium in fresh sea-water.

Observations

Are attractants stable? Suppose a star is placed in a test aquarium for a time and then removed; how long does the water retain its attraction for commensals?

Experiment No. 1. A host Evasterias was placed in non-circulating aerated sea-water overnight. After 18 hours the starfish was removed. The water in this aquarium was kept at 12.5° C. and continuously aerated for another 24 hours, at the end of which time it was tested:

No. of trials	16
No. of runs	10
No. of runs into Evasterias arm	4
No. of runs into sea-water arm	6
No. of failures	6
% failures	37%
No. of negative trials (6 plus 6)	12
% negative trials	75%
No. of *A. fragilis* used	8

At the end of these runs the starfish was returned to the test aquarium and three hours later, control tests were run:

No. of trials	11
No. of runs	10
No. of runs into Evasterias arm	9
No. of runs into sea-water arm	1
No. of failures	1
% failures	9%
No. of negative trials (1 plus 1)	2
% negative trials	18%

A comparison of the ratios of the number of runs into the Evasterias arm to the number of negative trials, both before and after the host was returned to the test aquarium (4/12 vs. 9/2), gives a χ^2 of 8.4 and a $P < .01$. The data indicate that the behavior of the commensals differed after re-introduction of the host; this agrees with the observed difference in behavior (increase in activity), there being a return of the normal observable response to the presence of the host in the system.

It would appear that water from which a host had been absent for twenty-four hours no longer retained its attraction for commensals.

In similar tests, water which had housed a star was shown to have lost its effectiveness five hours after removal of the star.

Experiment No. 2. Procedure was next reversed so that controls were run first and the star then removed; *i.e.*, a star was placed in the test aquarium, a number of trials made, the star removed and further trials immediately made. In this experiment the starfish was removed simply by lifting it from the test aquarium. However, considerable difficulty was encountered in removing the starfish, which clung firmly to the walls of the aquarium. Removal resulted in the tearing off of a number of tube feet which remained attached to the aquarium walls. The effect of such removal was immediate and dramatic.

Control:

No. of trials	18
No. of runs	18
No. of runs into Evasterias arm	16
No. of runs into sea-water arm	2
No. of failures	0
% failures	0%
No. of *A. fragilis* used	6

Host removed:

No. of trials	6
No. of runs	0
No. of failures	6
% failures	100%

This effect was so immediate, the behavior of the worms being so completely altered (lack of activity, reversal of position), that the host was returned to the apparatus and the testing continued. Re-introduction of the star at this point *did not* alter the negative behavior of the worms, even after the passage of 2–3 hours:

No. of trials	12
No. of runs	3
No. of runs into Evasterias arm	0
No. of runs into sea-water arm	3
No. of failures	9
% failures	75%

In this experiment, great care was taken so that no other factor was involved than the difficult removal of the host. The authors feel convinced that the immediate change in the behavior of the commensals was effected by removal of the host alone. Could the same experiment be conducted so that removal of the host could be accomplished without as much disturbance to it?

Experiment No. 3. This experiment resembled the preceding except that

when the host star was removed, it was given a quick and sudden shock so that it came freely and easily from its position in the aquarium.

Control:

No. of trials	19
No. of runs	19
No. of runs into Evasterias arm	18
No. of runs into sea-water arm	1
No. of failures	0
No. of negative trials (1 plus 0)	1
% negative trials	5%

Host removed:

No. of trials	19
No. of runs	16
No. of runs into Evasterias arm	13
No. of runs into sea-water arm	3
No. of failures	3
No. of negative trials (3 plus 3)	6
% negative trials	31%

In this experiment the effect of removal of the host on the behavior of the worms was not as marked as in the preceding one; it would appear that if the host is easily and delicately removed, some attraction for commensals remains in the water. A comparison of the two proportions of choice above (18/1 vs. 13/3) shows no statistically significant difference, but the appearance of a number of failures shortly after the host was removed and the observed decrease in activity and movement of the worms when on trial indicated that some change had occurred. It could not be determined whether this was the result of extreme instability of the attractant or of the slow dilution of whatever attractant remained in the test aquarium, occurring when it was necessary at the beginning of every trial to equalize the pressure in the arms of the Y by balancing the water in the two aquaria (*cf.* Davenport, 1950).

In view of the difficulty of removing the host from the test aquarium without negating the response, and because of the relatively short time that water which had housed the host retained its attraction, it appeared at this point difficult to design experiments to determine something about the nature of the attractant by testing its heat-stability, solubility in ether or alcohol, adsorption on charcoal, etc. One experiment, however, was conducted which gave some evidence as to the physical nature of the attractant.

Experiment No. 4. A single host Evasterias was placed in sea-water inside a ten-inch Visking Corp. dialyzing bag. This was left open at the top and in turn suspended in sea-water in the test aquarium. Bubblers were placed both inside and outside the dialyzing bag, giving thorough aeration and mixing. After the passage of two hours, trials were commenced:

No. of trials	12
No. of runs	3
No. of runs into Evasterias arm	1
No. of runs into sea-water arm	2
No. of failures	9
No. of negative trials (9 plus 2)	11
% negative trials	91%
No. of *A. fragilis* used	6

At the end of these tests which clearly indicated no attractant to be present outside the bag, the bag was carefully split open with a razor and the contained sea-water and starfish allowed to escape into the test aquarium:

No. of trials	12
No. of runs	12
No. of runs into Evasterias arm	12
No. of runs into sea-water arm	0

This experiment would indicate that the attractant does not readily diffuse through the dialyzing bag and that therefore it is probably not a relatively simple molecule.

Experiment No. 5. In an effort to determine whether the attractant may diffuse through such a membrane only very slowly, on the day following the preceding experiment the same starfish was placed in a bag and then suspended in the test aquarium for eleven hours before runs were made:

No. of trials	12
No. of runs	4
No. of runs into Evasterias arm	2
No. of runs into sea-water arm	2
No. of failures	8
No. of negative trials (8 plus 2)	10
% negative trials	83%
No. of *A. fragilis* used	6

The contents of the bag and the starfish were then released into the test aquarium:

No. of trials	13
No. of runs	7
No. of runs into Evasterias arm	4
No. of runs into sea-water arm	3
No. of failures	6
No. of negative trials (6 plus 3)	9
% negative trials	69%

This experiment gave no indication whatever as to how much of the attractant had diffused after the passage of eleven hours, since the performance of the commensals after the bag had been opened into the test aquarium was as negative as it had been before the bag was opened. However, the failure to respond after the opening of the bag was in itself interesting. In view of the fact that many control starfish had given positive series of runs after isolation in non-circulating, aerated sea-water for as long as 15 hours, the experiment gave evidence that suspension of a star in a bag for as long as 11 hours, in spite of constant aeration and no appreciable rise in temperature, constituted some disturbance which either prevented release of the attractant by the star or caused the star to produce substances which masked the effect of the attractant.

Can the source of the attractants in the host be localized?

Experiment No. 6. Is the integument of Stichopus the source of the attractant for *A. pulchra?* A single Stichopus was completely eviscerated and the oral and aboral regions removed, leaving a large rectangular strip of integument including the longitudinal musculature. This was thoroughly washed in fresh running sea-water in order to remove traces of the visceral contents and then placed overnight

in aerated but non-circulating sea-water at approximately 12.5° C. Both immediately prior to testing and at the end of the experiment some hours later, the integumental strip was still fresh and responded to stimulation or stretching by contraction.

No. of trials	41
No. of runs	23
No. of runs into Stichopus arm	12
No. of runs into sea-water arm	11
No. of failures	18
% failures	44%
No. of *A. pulchra* used	12

Experiment No. 7. In this experiment, a check on the preceding, the integuments of three large Stichopus selected at random were washed for some seven hours in fresh running sea-water to insure removal of any contaminants from the viscera, and in the hope that injury substances might be eliminated. The integumentary strips were placed in non-circulating, aerated sea-water at 12.5° C. overnight before testing. Both prior to and at the end of the experiment the three strips were fresh and responded to stimulation by contracting.

No. of trials	15
No. of runs	6
No. of runs into Stichopus arm	5
No. of runs into sea-water arm	1
No. of failures	9
% failures	60%
No. of *A. pulchra* used	6

Combining the data from experiments 1 and 2:

No. of trials	56
No. of runs	29
No. of runs into Stichopus arm	17
No. of runs into sea-water arm	12
No. of failures	27
% failures	48%

It can be seen in the above data that in these two experiments the distribution into the two arms did not significantly differ from a random one, while almost half the trials resulted in failures. It would appear from these experiments that *A. pulchra* will not respond to washed, eviscerated host integument.

Experiment No. 8. *A. pulchra* is sometimes found within the oral aperture of Stichopus; does the digestive system of the host produce an attractant? The entire viscera of a single large Stichopus was removed, remaining attached to an integumentary ring including the tentacles. This preparation was thoroughly washed, isolated in aerated, non-circulating sea-water overnight and tested.

No. of trials	25
No. of runs	13
No. of runs into Stichopus arm	2
No. of runs into sea-water arm	11
No. of failures	12
% failures	48%
No. of negative trials (11 plus 12)	23
% negative trials	92%
No. of *A. pulchra* used	12

From the proportion of runs into the Stichopus arm to the number of negative trials (2/23), it would appear that the commensals were repelled by the preparation, indicating, perhaps, the presence of digestive juices or injury products.

Experiment No. 9. Does the integument of Evasterias produce an attractant for *A. fragilis?* A freshly collected Evasterias was opened along the aboral surface of the arms and the entire viscera removed. This preparation was washed for two minutes in fresh running sea-water and tested.

No. of trials	26
No. of runs	15
No. of runs into Evasterias arm	9
No. of runs into sea-water arm	6
No. of failures	11
% failures	42%
No. of *A. fragilis* used	6

It appears from the above data that the cleaned, eviscerated integument of a host Evasterias has no attraction for commensal *A. fragilis.*

Experiment No. 10. A. fragilis are often found lying close to the mouth of the host star; could the digestive system give rise to the attractant substance? The entire digestive systems of three host stars selected at random were removed, ground up together in a small quantity of sea-water in clean sand and the preparation placed in the test aquarium.

No. of trials	18
No. of runs	6
No. of runs into Evasterias arm	1
No. of runs into sea-water arm	5
No. of failures	12
% failures	66%
No. of negative trials (5 plus 12)	17
% negative trials	94%

The very high percentage of negative trials indicates that the preparation repelled the commensals.

The above experiments (nos. 6–10) gave evidence that it will be difficult to localize the source of the attractants by dissection techniques. However, in the latter part of the summer, after experiments to determine the source of attractants had been conducted, it became apparent that for some unknown reason host-species individuals (both Evasterias and Stichopus) were beginning to show considerable variation insofar as attraction for commensals was concerned. Occasionally, specimens taken from the live-boxes as well as some freshly collected individuals exhibited no attraction whatever, in spite of appearing perfectly healthy and normal. Ideally, therefore, Experiments 6–10 should have been conducted with material dissected from whole animals with which immediately preceding control runs had been successfully made. Lack of time prevented repetition of the experiments using this control. However, as has been stated, Experiments 7 and 10 were controlled to the extent of using three preparations selected at random, while in Experiments 8 and 10, production of attractants by viscera, if still occurring, could not be demonstrated, since the preparations clearly repelled commensals.

Experiment No. 11. The circumstance of discovering a locality where large numbers of immature Evasterias could be found made possible an experiment to

give evidence as to whether the young stars exert as strong an attraction for commensals as do older ones. Forty-one small Evasterias weighing a total of approximately 517 grams were placed in non-circulating, aerated sea-water overnight. The commensals used were taken from large stars.

No. of trials	13
No. of runs	13
No. of runs into Evasterias arm	6
No. of runs into sea-water arm	7
No. of failures	0
No. of *A. fragilis* used	6

Tests of commensals against sea-water only (Davenport, 1950) showed that out of 21 trials, 8 were failures and the commensals were very inactive. In the above experiment the absence of failures and the observed behavior of the worms indicated the presence of attractant, but the random distribution into the arms would seem to indicate that concentrations may not have been high enough to have a strong positive effect on the choice of the commensals.

The Genus Halosydna

Material

The commensal partnership exhibited by the terebellid *Amphitrite robusta* (Johnson) and the polynoid *Halosydna brevisetosa* Kinberg would appear to be a most valuable one for study. In the first place the animals can very readily be collected at medium low tides at numerous localities near the laboratories. The material used in the following experiments was found under boulders at Jones Beach, San Juan Island; Minnesota Reef, San Juan Island; a point on Wasp Passage, Orcas Island; and a point near the village of Olga, Orcas Island. Almost any boulder-strewn intertidal area in which there is a soft muddy-sand substrate is likely to harbor numbers of these interesting terebellids and their commensals. Often under the same boulders and in *immediate contact* with the tubes of Amphitrite which house both host and commensal can be found the tubes of the green terebellid *Eupolymnia crescentis* Johnson with its commensal crabs (*Pinnixa* sp.). No Halosydna were found commensal in these neighboring Eupolymnia tubes, nor have they ever been so described. Miss Pettibone (1947) has also found the scale worm in association with *Thelepus crispus* Johnson in Puget Sound. At the same time, free-living and morphologically different *Halosydna brevisetosa* can be readily collected among the byssus threads of mussels and among the roots of the basket grass, Phyllospadix (rocky coast of west side of San Juan Island; Iceberg Point, San Juan Island, etc.).

Here, therefore, are forms in which it should be possible to design experiments comparing the behavior of free-living and commensal members of the same species in relation to the two host forms, and in relation to relatives of the host forms often associated with them but never housing the commensals.

Initially it was imperative to determine whether the same type of chemical attraction existed between Amphitrite and Halosydna as had been demonstrated in echinoderm-annelid partnerships.

Methods

Commensal teams of these animals were either kept all together in a large open white tray of circulating sea-water, or host and partner were kept together in finger-bowls immersed in sea-water in a large tray. It was found that as long as there was constant and rapid water change and aeration, the animals would stay in good condition for many days; experiments were conducted, however, with recently collected material. The method of conducting experiments was identical to that used for the echinoderm-annelid partnership.

Observations

In two separate experiments, using different animals, it was found impossible to demonstrate any attraction for commensal Halosydna by sea-water containing host *Amphitrite robusta*.

Experiment No. 1. Nineteen Amphitrite were placed in non-circulating aerated sea-water overnight.

No. of trials	58
No. of runs	50
No. of runs into Amphitrite arm	26
No. of runs into sea-water arm	24
No. of failures	8
No. of Halosydna used	8

Experiment No. 2. Eighteen Amphitrite were placed in non-circulating aerated sea-water overnight.

No. of trials	60
No. of runs	59
No. of runs into Amphitrite arm	35
No. of runs into sea-water arm	24
No. of failures	1
No. of Halosydna used	6

In this experiment, after 42 trials had been run giving a random distribution, a large number of pieces of the sandy tubes of the hosts were placed in the test aquarium with them. The presence of this tube material in no way altered the subsequent behavior of the commensals, their distribution in the arms of the Y still being random.

Combining the data for Experiments 1 and 2:

No. of trials	118
No. of runs	109
No. of runs into Amphitrite arm	61
No. of runs into sea-water arm	48
No. of failures	9

In these experiments, it was clear that water coming from a number of host terebellids had no attraction for the commensal Halosydna. It therefore appeared necessary to attack the problem of the agency governing this partnership from some other direction; perhaps chemical factors are not involved. It was impossible to do more than to initiate this work with some brief and qualitative experiments.

It was first determined that although slightly negative to light, commensal Halosydna are not strongly negatively phototactic. A glass aquarium was painted an opaque black, leaving a small transparent window at one end. A lamp placed at this window enabled one to flood the bottom of the tank with light from one end. Worms were observed through a dark but transparent glass cover. If a number of Halosydna were placed in the aquarium with the light on, they were observed to make more or less random movements and after the passage of some minutes, the majority congregated at the dark end of the aquarium.

Observations were also made on the behavior of the commensals when placed in open white trays with a host animal. Such commensals moved slowly about the tray in a random manner until they came in contact with one of the long extended tentacles of the host. This contact caused commensals to move directly toward the Amphitrite along the tentacle until they came in contact with the body of the host, whereupon they wrapped themselves around the body of the host. This response after contact with the tentacle was most marked.

It remains to investigate whether similar responses of free-living Halosydna to Amphitrite and of commensal Halosydna to non-host terebellids exist.

Conclusions

Considering the experiments with the echinoderm-annelid partnerships as a whole, it would appear that a rather tenuous bond exists between host and commensal.

Initially, it is clear that the attractant from Evasterias is relatively unstable. Experiments gave evidence that this substance did not readily diffuse through dialyzing membranes.

A number of different experiments seemed to indicate that any disturbance to the host is likely to negate the response of commensals. Violent removal of the host from the test aquarium apparently brings about the release of substances which not only negate the response but actually repel the commensals. Suspension of a host in a dialyzing bag for eleven hours negated the response of worms to this host after the host had been released. It seemed impossible to demonstate any response of commensals to parts of their hosts. These results are quite in agreement with those obtained (Davenport, 1950) in tests on the Luidia-Arctonoë partnership in which it was impossible to confine a host Luidia in a test aquarium without autotomy of arms occurring, and in which failure of commensals to respond to the host was attributed to the consequent release of repelling injury substances, as indicated by the high number of negative trials (27/35).

Finally, the tenuity of the bond is also indicated by the variation in the power of attraction among individual Evasterias and Stichopus, which first became evident about the middle of the summer during which these investigations were made. Undoubtedly the inability of hosts to attract commensals resulted in some cases from confinement under slightly abnormal conditions in live-boxes, for some individuals that had earlier in the summer given strong series of positive runs later lost their attraction. However, the failure of a few freshly collected hosts to attract commensals is unexplained. This may have been the result of unknowing adverse treatment in handling, but perhaps there is fluctuation in the ability of hosts to attract.

Two possibilities concerning the agency governing the echinoderm-annelid relationships would seem to exist. One is that the specificity of the host-commensal relationship may depend upon the conditioning of commensals to a single, relatively unstable attractant, which is rapidly altered, or the effect of which is easily masked by other substances produced by disturbance or damage to the host animal. It may be possible, though it does not seem reasonable to assume it, that the production of the substance on which specificity depends would suddenly cease if hosts were dissected or disturbed or simply kept under conditions differing so little from natural ones as those in the live-boxes. It would seem more likely that attractants are readily masked by other substances, although this was certainly not the case in Welsh's (1930) experiments in which clam tissue retained its effect on the phototaxis of commensal mites even after decomposition at 37.5° C. for a week. In this particular partnership Welsh's experiments indicated the presence of stable and powerfully-acting agents which could not be altered or masked by decomposition; from this he reasoned that the effective substances might be decomposition products of the mucus or other proteins from the gills.

A second possibility for the echinoderm-annelid partnership should be kept in mind, although for it there is admittedly little experimental evidence at this time. It is possible that commensals may be conditioned to a pattern of chemical stimuli from the host and that since this pattern is made up of the sum of a number of factors it is therefore a delicate one which may be altered by a change in any one of the factors with the result that the response of commensals is negated.

At any rate the relative instability of attractants as well as the ease with which their effect may be negated by any disturbance to the host will obviously make more difficult an analysis of their physical and chemical characteristics.

In the echinoderm-annelid relationships the adaptive value of this conditioning to a substance or pattern of substances which is relatively unstable and which may be easily masked is clear. Both Evasterias and Stichopus are generally found in association with numbers of their fellows. Any circumstance bringing about dangerously abnormal conditions for the host (attack and autotomy, evisceration, etc.) may break the bond for the commensal and conceivably allow it to search out another normal host. To test this hypothesis experimentally should be possible.

The experiments with Halosydna and Amphitrite need not be taken to indicate that chemical factors are unimportant as agents of this commensal relationship. It is clear that no response could be demonstrated with the Y-tube apparatus. The response of the commensals to the tentacles of the terebellid may indicate the presence of a relatively insoluble attractant closely bound to the tentacles of the host, or the response may be purely tactile, chemical factors not being involved at the stage of the partnership experimented with. At any rate, these experiments, as well as those with the echinoderm-annelid partnerships, emphasize the importance of thorough investigations of the life-histories of commensals in relation to life-histories of hosts, in an effort to determine time and manner of adaptation to hosts, etc. In this regard Dr. Pettibone (1947) has found young Halosydna not more than 7–9 mm. long in association with young Thelepus; she believes that commensalism in Halosydna may start early and that host and commensal grow up together. This is in agreement with the observations of the authors, who found only large Halosydna commensal with the large Amphitrite of the collecting station near Olga. However, in the Evasterias-Arctonoë relationship, Miss Pettibone and the writers have often

found small (4–5 mm. in length) commensals, sometimes in numbers, on large hosts and often on one host commensals of differing growth stages. Greater numbers of large stars possess commensals than do immature stars; occasionally, however, one will find commensals from 3–4 cm. long on hosts that are not more than 4 cm. in diameter. In such a partnership the commensal will take up space in the ambulacral groove almost all the way across the starfish. It would therefore appear that in the echinoderm-annelid relationship a number of generations of commensals may pass their life on the same host; we also find commensals of differing growth stages on Stichopus. However, as has been said, although one may often find on a single Evasterias a number of young commensals, one rarely if ever finds more than one large Arctonoë on a starfish.

As yet no evidence (of brooding young, etc.) has appeared that would lead the writers to believe that the entire life-history of the worms is passed on the host. It is possible that the free-living larval stage may be of short duration. Ultimately, it must be determined at what stage and by what mechanism commensals find the host.

Summary

1. Water from an aquarium from which a host *Evasterias troschelii* has been removed 24 hours before no longer possesses an attraction for commensal *Arctonoë fragilis*. Similar results were obtained five hours after removal.

2. Water from an aquarium from which a host Evasterias has been removed with difficulty, so that tube feet were torn from it, has no attraction for commensals almost immediately after removal of the host. Such water appears to repel them.

3. Water from an aquarium from which a host Evasterias has been removed with comparative ease (involving less disturbance to the starfish) retains its attraction for commensals immediately after removal, but shortly thereafter the attraction appears to weaken.

4. If a host Evasterias is suspended in a test aquarium in a dialyzing bag for two hours, at the end of this time not enough attractant has passed through to attract commensals.

5. Experiments to determine whether attractant will diffuse through a dialyzing bag in effective concentrations overnight may be unsuccessful because the abnormal conditions inside the bag affect the host in such a way that the response of commensals is negated even after the host has been released from the bag into the test aquarium.

6. Washed eviscerated integument of *Stichopus californicus* does not appear to attract its commensal, *Arctonoë pulchra*.

7. Washed eviscerated integument of Evasterias does not appear to attract its commensal.

8. Washed viscera of Stichopus appears to repel its commensal.

9. Ground-up washed preparations of viscera of Evasterias appear to repel its commensal.

10. Immature Evasterias do not exert as strong an attraction on commensals as adults, even after compensation has been made for difference in weight.

11. No chemical attraction for its commensal, *Halosydna brevisetosa,* paralleling that observed in specific echinoderm-annelid partnerships can be demonstrated in the terebellid *Amphitrite robusta,* in spite of the fact that the scale worm is limited to this and one other terebellid host.

12. Commensal Halosydna are neutral to light or mildly negatively phototactic. A positive tactile response to the tentacles and body of the host was found in these worms.

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THE EFFECT OF ILLUMINANCE ON THE REVERSAL TEMPERATURE IN THE DRONE FLY, ERISTALIS TENAX

WILLIAM L. DOLLEY, JR., AND JOHN D. WHITE

Department of Biology, University of Buffalo, Buffalo, N. Y.

For over seventy-five years physiologists have attempted without success to unravel the mechanisms involved in the change of sign of reaction of organisms to light. In the reversal an organism which is normally photopositive, moving toward the light, becomes negative and avoids the light, or a normally photonegative organism, moving away from the light, becomes positive. Recently a study of this phenomenon in the drone fly, *Eristalis tenax,* was begun and additional facts have been discovered. According to Dolley and Golden (1947a), in an illuminance of 700 foot candles Eristalis is highly photopositive within a temperature range between approximately 10° and 30° C. Outside these limits it is highly negative. Above 30° C., the temperature at which Eristalis changes its reaction to light depends upon the sex and age of the flies. Females cease their positive reaction to light and become negative at a higher temperature than do males, and the younger the fly, the higher the temperature at which it becomes negative. Furthermore, according to Dolley and Golden (1947b), in an illuminance of 700 f. c., a correlation exists between the temperature at which Eristalis changes the sign of its reaction to light (its reversal temperature) and the temperature at which it dies (its lethal temperature). In general, the higher the reversal temperature, the higher the lethal temperature.

Since nothing was known about the relation between illuminance and reversal temperatures in any organism, a study of this relation in Eristalis was made. The results of this study are presented in this paper.

Materials and Methods

The apparatus used (Fig. 1) consists of a box (50 × 35 × 34 cm.) made of 6.3 mm. plywood. There are two main compartments, a light one, A, and a dark one, B. The two compartments, A, 6.25 cm. deep, and B, 15 cm. deep, are separated by a wooden slide, a, which can be raised. This slide is painted white on the side toward the light compartment. Above the light compartment are two sliding glass panels, b, c, 6.3 cm. apart. A thermometer, d, is inserted through a hole, e, at the level of the white floor, f.

The light compartment is lined with white cardboard on the bottom, two sides, and one end. The dark compartment is lined with dull black cardboard and has a removable wooden cover, g, through the center of which a thermometer, h, is inserted.

Beneath the detachable floor, i, of the light compartment are placed five wooden blocks, j (5 × 3 × 3 cm.), for support. These blocks rest on a sheet of galvanized metal, k.

The floor of the dark compartment, l, is a piece of black paper, beneath which are sheets of smooth cardboard, corrugated cardboard, and galvanized metal, m.

Below the metal sheets are two heat chambers, C, D, 12.5 cm. deep, lined with corrugated cardboard, n, and asbestos sheeting, o. The heat is supplied by two 100-watt Mazda lamps, p, each wired separately.

A 300-watt Mazda reflector spot lamp, q, is suspended over the center of the light compartment. By appropriate resistance the voltage of this lamp was kept

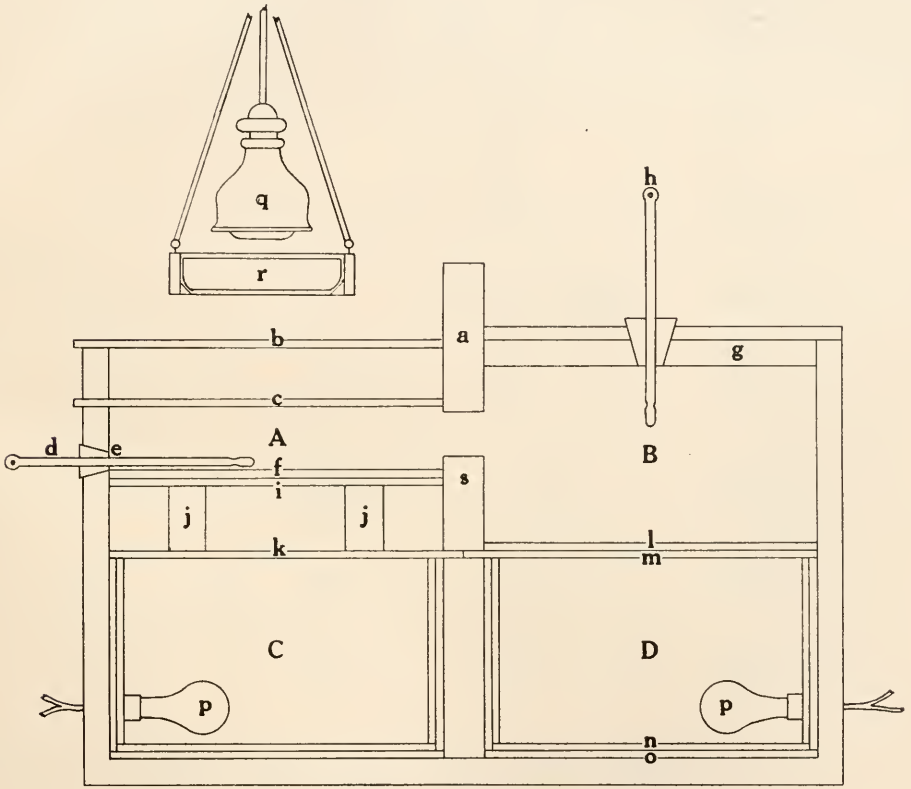

FIGURE 1. Sectional view of the apparatus used. See text.

constant at 105. A round glass dish, r, 23.7 cm. in diameter, containing distilled water to a depth of 2.5 cm., is beneath the lamp. This unit, q, r, can be raised or lowered to change the illuminance on the white floor, f. This illuminance was recorded by a Weston exposure meter.

In these experiments only young flies, 5 to 16 days old, were used. They were raised at room temperature, approximately 25° C., according to methods essentially similar to those previously described (Dolley *et al.*, 1937, p. 410), except that the larvae were cultured in cow manure.

The temperature at which the flies became negative to light in a known illuminance was determined. This was done for 270 males and 270 females in each of

the five illuminances: 250, 600, 800, 1200, and 1600 f. c. Only one observation was made on each fly.

All experiments were performed in a dark room. Before each experiment a record was made of the relative humidity of the air in the room. The same lamp was used in all of the experiments in all of the illuminances tested except those in 1200 f. c. The lamp used at this illuminance was, however, identical with the other one, so far as could be determined. A typical experiment was made as follows. The

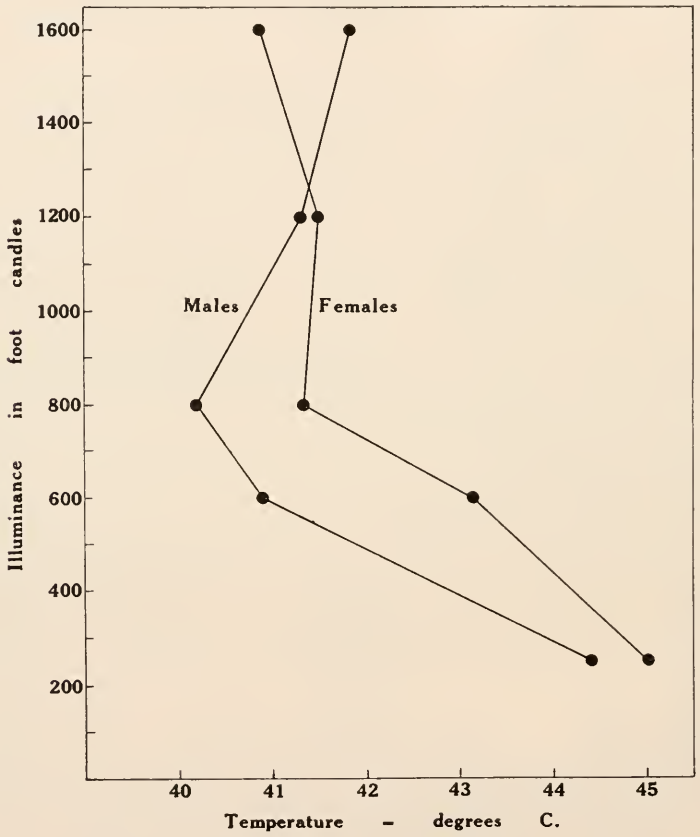

FIGURE 2. Graph showing the effect of illuminance upon the temperature at which reversal in reaction to light in Eristalis occurs. Note that the mean temperatures of the reversals decrease to a minimum at 800 f. c. In the males this decrease is followed by an increase, while in the females it is not. See text and Table I.

glass dish, r (Fig. 1), was filled with distilled water and the illuminating unit, q, r, was so adjusted that the illuminance desired was obtained on the floor, f, of the light compartment, A. The temperature in the dark compartment, B, was raised to between 35° and 37° C., that in the light compartment to 28° C. Throughout the experiment the temperature in the dark compartment was 6 to 9 degrees higher than that in the light compartment. Approximately 20 flies of the same sex, and with unclipped wings, were placed in the light compartment by sliding back the glass plates, b, c. These plates were replaced; the heating unit under the light chamber

was turned on, and the center slide, a, was raised, making an opening (18 × 4 cm.) connecting the two chambers.

As the temperature in the light chamber rose, the flies, becoming restless, crawled and flew about. Soon individuals moved out of the light into the darkness of the dark chamber. A fly was considered to have reversed when it had passed completely beyond the center ridge, s. When this occurred the investigator recorded to the nearest 0.5° the temperature in the light compartment. The flies followed one another, and one by one entered the dark compartment. Frequently a fly returned to the light compartment after a few minutes in the dark, and then after a few seconds returned again to the dark compartment. Sometimes a given fly made three or four such successive reversals. The temperature at which the final reversal of a given fly took place was recorded and considered one observation. After

TABLE I

The effect of illuminance on the temperature at which reversal in reaction to light in Eristalis occurs. See text.

Illuminance in foot candles	Sex	Mean temperature in degrees centigrade ± standard error	Standard deviation ± standard error
250	Male	44.40 ± .023	3.702 ± .159
	Female	45.011 ± .241	3.964 ± .171
600	Male	40.917 ± .228	3.744 ± .161
	Female	43.133 ± .210	3.445 ± .148
800	Male	40.194 ± .182	2.983 ± .128
	Female	41.339 ± .220	3.622 ± .156
1200	Male	41.317 ± .187	3.075 ± .132
	Female	41.506 ± .223	3.660 ± .158
1600	Male	41.828 ± .252	4.148 ± .179
	Female	40.872 ± .247	4.056 ± .175

approximately 35 minutes all the flies had reversed and entered the dark compartment.

Although the temperature throughout the light chamber was probably not uniform the reversal temperatures measured were very close to those where the flies were at a given instant. The thermometer measured the temperature on the floor of the chamber. During most of the periods of exposure the flies were in contact with this floor.

The thermometers used read to one degree. A check on their accuracy revealed that they read approximately 0.2° too high. This error, however, does not invalidate the general conclusions drawn, since it appeared in all the measurements made and is within the limits of error of the experiments.

RESULTS

The results obtained are given in Figure 2 and Table I.

Are the differences between the mean temperatures at which the male flies reversed in the various illuminances significant? According to Pearl (1940, p. 287), "the odds are 369.4 to 1 against the occurrence of a deviation in either the plus or minus direction as great or greater than 3 × S. E. These are long odds, and are conventionally regarded as amounting to practical certainty." The differences between the means at 250 and 600, 600 and 800, 800 and 1200, and at 1200 and 1600 f. c. are, respectively: 9 +, 2 +, 4 +, and 1 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: over 400,000,000,000; 79; over 15,770; and 8 to 1. It is therefore evident that the differences between the means at 250 and 600 and at 800 and 1200 f. c., are clearly significant, while the difference between the means at 600 and 800 f. c., is probably not significant. That between 1200 and 1600 f. c., is certainly not significant. Consequently, it is clear that as the illuminance increased from 250 to 1600 f. c., the mean temperatures at which the male flies reversed decreased to a minimum at 800 f. c., and then increased.

As to the females, the differences between the means at 250 and 600, 600 and 800, 800 and 1200, and at 1200 and 1600 f. c., are, respectively: 5 +, 5 +, less than 1, and 1 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: over 1,744,000; over 1,744,000; less than 1; and 16 to 1. It is obvious that the differences between the means at 250 and 600 and at 600 and 800 f. c., are clearly significant, while the differences between the means at 800 and 1200 and at 1200 and 1600 f. c. are certainly not significant.

The data presented in Figure 2 and Table I do not permit a definite conclusion as to the reversal temperatures of the females in illuminances of 1200 and 1600 f. c. Apparently the reversal temperature at 1200 f. c. was slightly higher than that at 800 f. c., and the reversal temperature at 1600 f. c. was less than that in the two preceding illuminances. It is, however, probable that there is an error, for some unknown reason, in the mean temperature recorded for female flies at 1600 f. c., since male flies never reversed at higher temperatures than female flies in any other illuminances tested, neither those used in this work, nor that used by Dolley and Golden (1947a), 700 f. c. Moreover, the difference between the mean temperatures recorded for male and female flies at 1600 f. c. is of questionable significance, as is explained later. With the data available it is impossible to decide whether the mean temperature recorded for female flies at 1200 f. c. is erroneous or not.

The differences between the means of the two sexes at the various illuminances: 250, 600, 800, 1200, and 1600 f. c., are, respectively: 1 +, 7 +, 4 +, less than 1, and 2 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: 13; over 400,000,000,000; over 15,770; 1; and 143 to 1. It is therefore evident that the differences between the means of the two sexes in illuminances of 600 and 800 f. c., are significant. The differences between the means in the other three illuminances are either not significant or of questionable significance. Consequently, it is obvious that the female flies reversed at a higher temperature than the male flies in illuminances of 600 and 800 f. c. A similar sexual difference was reported by Dolley and Golden (1947a) in Eristalis in an illuminance of 700 f. c.

Discussion

The results presented in this paper show that as the illuminance rises from 250 to 1600 f. c., the reversal temperature of Eristalis decreases to a minimum at 800 f. c. In the males this decrease is followed by an increase. In the females further increase in the illuminance causes no significant change in the reversal temperature. Since, according to Dolley and Golden (1947b), there is a correlation between reversal and lethal temperatures in Eristalis in 700 f. c., the mechanisms involved in both phenomena are related.

The results obtained are not due to differences in relative humidity or in the duration of exposure of the insects to heat. There was variation in these factors in the thirteen or more experiments performed at various times during three years on flies of each sex in each of the illuminances used. Yet when the values of these factors in all the experiments are compared, there is no significant difference in the average value of these factors in the various illuminances.

Summary

1. Observations were made on 2700 young flies in ascertaining the temperature at which *Eristalis tenax* becomes negative to light in the following illuminances: 250, 600, 800, 1200, and 1600 f. c.

2. As the illuminance increases from 250 to 1600 f. c., the mean temperatures at which the male flies reverse decrease to a minimum at 800 f. c., and then increase.

3. As the illuminance increases from 250 to 1600 f. c., the mean temperatures at which the female flies reverse decrease to a minimum at 800 f. c. Further increase in the illuminance causes no further significant change in the mean reversal temperature.

4. In illuminances of 600 and 800 f. c., the temperature at which Eristalis changes in its reaction to light depends upon the sex of the fly. Females cease their positive reaction to light and become negative at higher temperatures than do the males. This difference between the sexes does not exist at the following illuminances: 250, 1200, and 1600 f. c.

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THE EFFECT OF ILLUMINANCE ON THE LETHAL TEMPERATURE IN THE DRONE FLY, ERISTALIS TENAX

WILLIAM L. DOLLEY, JR., AND JOHN D. WHITE

Department of Biology, University of Buffalo, Buffalo, N. Y.

Although heat injury in organisms has aroused the interest of many able physiologists, the mechanisms involved are still unknown. According to Bělehrádek (1935), various authors have suggested that heat death is due to (1) the coagulation of proteins, (2) the destruction of enzymes, (3) asphyxiation, caused by the inability of the oxygen supply to keep pace with the needs caused by increased metabolic rate, (4) intoxication produced by certain products, and (5) a change in the physical state of lipids.

Recently a study of this phenomenon in the drone fly, *Eristalis tenax,* was begun and as a result additional facts have been discovered. According to Dolley and Golden (1947), in an illuminance of 700 foot candles, the temperature at which Eristalis dies, the lethal temperature, depends upon the sex and age of the flies. The lethal temperature is higher for females than for males of the same age, and it is higher for young flies than for old flies. Moreover, according to these authors, there is a correlation between the lethal temperature and the reversal temperature in an illuminance of 700 f. c. In general, the higher the reversal temperature, the higher the lethal temperature.

Since nothing was known about the relation between the lethal temperature and illuminance in any organism, a study of this relation in Eristalis was made. The results obtained are presented in this paper.

Materials and Methods

The apparatus and methods used were those described previously (Dolley and White, 1951) with the following exceptions: the same lamp, that used in all the illuminances but one mentioned in the previous paper, was employed for all illuminances used in this work; the dark chamber (Fig. 1, B, previous paper) was not used; the slide, a, was not raised; and the procedure was modified as described in the three following paragraphs.

The temperature at which the flies died in a known illuminance was determined. This was done for 270 males and for 270 females in each of the five illuminances: 250, 600, 800, 1200, and 1600 f. c.

Approximately thirty young flies of the same sex were placed in the light chamber, A (Fig. 1, previous paper). As the temperature rose, the flies becoming restless, crawled and flew about. Soon the animals, one by one, fell upon their backs, whirled around for a few moments, and then became quiet, except for a flexing and extension of the muscles of one or more legs, and extension and withdrawal of the proboscis. This movement continued for a brief interval. When all perceptible movement in a fly ceased, the temperature at which this occurred

was recorded as the lethal temperature of this specimen. In quick succession the other flies died and their lethal temperatures were recorded.

Often certain flies crawled into a corner near the top of the chamber where the temperature was lower than at the bottom. When this occurred, the thermometer, d, placed in a hole near the bottom of the chamber was removed, and by means of a wire inserted through this hole the fly was dislodged. The thermometer was then replaced. After approximately 45 minutes all the flies had died. They were removed, the heating unit disconnected, and the box was allowed to cool. The heating unit was again connected, and, when the temperature in this chamber had again become about 28° C., another lot of flies was placed in the chamber and the process repeated.

Although the temperature throughout the light chamber was probably not uniform the lethal temperatures measured were very close to those where the flies were at a given instant. The thermometer measured the temperature on the floor of the chamber. During most of the periods of exposure the flies were in contact with this floor.

Results

The results obtained are given in Figure 1 and Table I.

The mean temperatures in degrees centigrade at which the male flies died are given in Table I. The differences between the means at 250 and 600, 600 and 800, 800 and 1200, 1200 and 1600 f. c. and at 250 and 1600 f. c., are respectively: 8 +, less than 1, 1 +, 10 +, and 1 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: over 400,000,000,000; less than 1; 3; over 400,000,000,000; and 13 to 1. Evidently the differences between the means at 250 and 600 and at 1200 and 1600 f. c. are definitely significant, while the differences between the means at 600 and 800, 800 and 1200, and at 250 and 1600 f. c., are not significant.

Therefore, it is clear that as the illuminance was increased from 250 to 1600 f. c., the mean temperatures at which the male flies died decreased to a minimum at 600 f. c. and then increased to a maximum at 1600 f. c., and this mean temperature at 1600 f. c. was approximately the same as that at 250 f. c.

The mean temperatures in degrees centigrade at which the female flies died are given in Table I. The differences between the means at 250 and 600, 600 and 800, 800 and 1200, 1200 and 1600, and at 250 and 1600 f. c., are, respectively: 9 +, 1 +, 2 +, 10 +, and less than 1 times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: over 400,000,000,000; 16; over 267; over 400,000,000,000; and less than 1 to 1. Consequently, the differences between the means at 250 and 600 and at 1200 and 1600 f. c. are definitely significant; the difference between the means at 800 and 1200 f. c. is probably significant; and the differences between the means at 600 and 800 and at 250 and 1600 f. c. are not significant.

As the illuminance was increased from 250 to 1600 f. c., the mean temperatures at which the female flies died decreased to a minimum at 600 f. c., and then increased to a maximum temperature at 1600 f. c., about the same as that at which the flies died in 250 f. c.

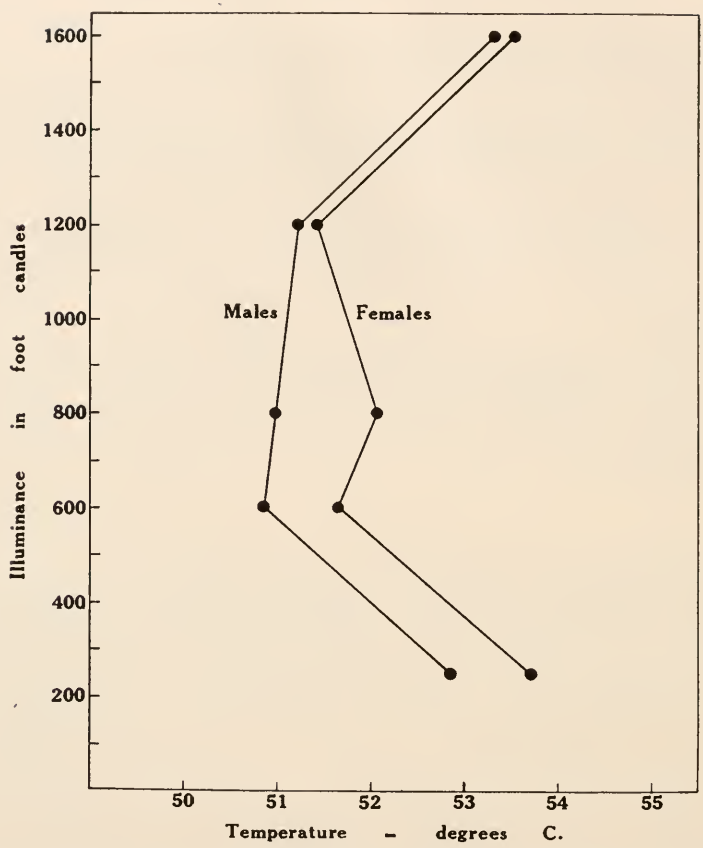

FIGURE 1. Graph showing the effect of illuminance on the temperature at which Eristalis dies. Note that the means of the lethal temperatures decrease to a minimum at 600 f. c., and then increase to a maximum at 1600 f. c. See text and Table I.

TABLE I

The effect of illuminance on the temperature at which Eristalis dies. See text.

Illuminance in foot candles	Sex	Mean temperature in degrees centigrade ± standard error	Standard deviation ± standard error
250	Male	52.956 ± .194	3.193 ± .137
	Female	53.811 ± .182	2.984 ± .128
600	Male	50.889 ± .162	2.658 ± .114
	Female	50.661 ± .152	2.50 ± .11
800	Male	50.994 ± .141	2.310 ± .099
	Female	52.089 ± .157	2.581 ± .111
1200	Male	51.222 ± .140	2.307 ± .099
	Female	51.428 ± .150	2.457 ± .106
1600	Male	53.417 ± .177	2.910 ± .125
	Female	53.65 ± .16	2.618 ± .113

The differences between the means of the two sexes in the various illuminances (250, 600, 800, 1200, and 1600 f. c.) are, respectively: 3 +, 3 +, 5, 1, and less than 1 times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively: over 726, over 1483, over 1,744,000, 2, and less than 2 to 1. It is obvious that the differences between the means of the two sexes in illuminances of 250, 600, and 800 f. c. are significant, while the differences in the other two illuminances are not significant. The female flies died at higher temperatures, therefore, than did the male flies in illuminances of 250, 600, and 800 f. c. This is in accord with the work of Dolley and Golden (1947).

Great confidence is felt in the results reported in this paper and in the preceding one on reversal temperatures. Essentially similar results were obtained in extensive preliminary experiments which were discarded because sources of light open to possible criticism were used.

As stated previously, Dolley and Golden (1947) reported a correlation between the reversal and lethal temperatures in Eristalis, in an illuminance of 700 f. c., the higher the reversal temperature, the higher the lethal temperature. This statement also holds true for illuminances of 250, 600, 1200, and 1600 f. c., but apparently does not at 800 f. c.; as the illuminance increased from 600 to 800 f. c., the mean temperature at which the flies reversed decreased (Fig. 2, Dolley and White, 1951), but there was no corresponding decrease in the mean temperature at which the flies died in these two illuminances (Fig. 1, this paper).

The results obtained are not due to differences in relative humidity or in the duration of exposure of the insects to heat. There was variation in these factors in the nine or more experiments performed at various times during three years on flies of each sex in each of the illuminances used. Yet when the values of these factors in all of the experiments are compared, there is no significant difference in the average value of these factors in the various illuminations.

Discussion

The results presented in this paper show that as the illuminance rises from 250 to 1600 f. c., the lethal temperature of Eristalis decreases to a minimum and then increases to a maximum. In other words, as the light energy to which the flies are exposed increases, the resistance of the organisms to heat decreases to a minimum and then rises to a maximum. These results are in harmony with the results reported by certain investigators on the physical changes induced by heat in certain organisms and in certain important constituents of organisms: proteins, lipids, and enzymes. According to Heilbrunn (1943, p. 78), in some types of protoplasm, as the temperature is raised, the protoplasmic viscosity increases and then decreases, *i.e.*, it goes through a maximum. Moreover, according to Ostwald (1913), as the temperature of a weak albumin sol rises, the viscosity first decreases, then sharply increases to a maximum, and then decreases again. The same author maintains that as the temperature of a lipid, isobutyric acid, in water, rises, the viscosity decreases slightly, then rises to a maximum, and then decreases. According to Sizer (1943), if enzyme reactions are exposed to rising temperature, the rate of the reactions increases to a maximum and then decreases.

Consequently, the curves describing the results given in this paper are strikingly similar in some respects to those curves reported previously by other investigators

recording the viscosity changes in certain proteins, lipids, types of protoplasm, and changes in rates of certain enzyme reactions, on exposure to rising temperature. The significance of this similarity awaits further work. The results presented for Eristalis do not seem to lend support to the asphyxiation or intoxication theories listed by Bělehrádek, but they are in harmony with the other three theories mentioned previously. Heat death in Eristalis, therefore, may be due to any one, two, or three of the following: viscosity changes in certain proteins and lipids, and change in rate of reactions of certain enzymes.

Summary

1. Observations were made on 2700 young flies in ascertaining the temperature at which Eristalis dies in illuminances of 250, 600, 800, 1200, and 1600 f. c.

2. As the illuminance increases from 250 to 1600 f. c., the mean temperatures at which the male and female flies die decrease to a minimum at 600 f. c., and then increase to a maximum temperature at 1600 f. c., about the same as that at which they die in 250 f. c.

3. In illuminances of 250, 600, and 800 f. c., the temperature at which Eristalis dies depends upon the sex of the fly. The females die at higher temperatures in these illuminances than do the males. This sexual difference is not present in illuminances of 1200 and 1600 f. c.

4. There is a correlation between the reversal and lethal temperatures in Eristalis in illuminances of 250, 600, 1200, and 1600 f. c. The higher the reversal temperature, the higher the lethal temperature. This correlation does not apparently exist in an illuminance of 800 f. c.

5. As the light energy to which Eristalis is exposed increases, the resistance of the organisms to heat decreases to a minimum and then rises to a maximum. Consequently, the curve describing these events resembles greatly those curves reported previously by other investigators recording the viscosity changes in certain proteins, lipids, and protoplasms, and changes in rates of certain enzyme reactions, on exposure to rising temperature.

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MOVEMENTS OF THE THORAX AND POTENTIAL CHANGES IN THE THORACIC MUSCLES OF INSECTS DURING FLIGHT [1]

KENNETH D. ROEDER

Department of Biology, Tufts College, Medford, Massachusetts

The wingbeat frequency of insects ranges from 5 per second in some butterflies up to 1000 per second in certain midges (Sotavalta, 1947). The most carefully investigated flight performance is that of Drosophila, which is capable of continuous flights of up to two hours at an initial wingbeat frequency as high as 300 per second which drops to not less than 100 per second as fatigue terminates the flight (Williams, Barness and Sawyer, 1943). This prodigious motor performance poses many questions regarding the metabolic fuel (Chadwick, 1947; Wigglesworth, 1949) and power output (Chadwick and Williams, 1949), and also demands a muscular mechanism capable of a cycle of excitation, contraction, and relaxation within an interval of three milliseconds.

In a recent study of excitation of the wing muscles of another fly, Calliphora, Pringle (1949) recorded a sequence of spike potentials during flight from electrodes inserted among the indirect flight muscles. Although similar in form and duration to muscle potentials recorded from other arthropods, these spike potentials showed a complete lack of synchrony with the wing movements, recurring in a rhythm of their own of one spike to every dozen or so wingbeats. Pringle concludes that each spike is the sign of the arrival of a motor nerve impulse whose action is to render the indirect flight muscles susceptible to the stimulus of stretch. Contraction of the vertical muscles stretches the horizontal muscles, and the myogenic oscillation thus established continues for as long as the requisite state of excitation is maintained by the arrival of motor nerve impulses.

This novel mechanism is in contrast to the situation encountered in a much less specialized insect, the roach *Periplaneta americana*. Excitation of muscles in the leg (Pringle, 1939) and thorax of this insect (Roeder and Weiant, 1950) is indicated by the appearance of a spike potential preceding each contraction. Although the loading of the muscle was not controlled, Roeder and Weiant recorded a latency of 3.0 milliseconds, duration of muscle potential of 4–5 milliseconds, and contraction-relaxation time of 20–25 milliseconds. Since the wing beat frequency of the roach is only 20–30 per second at room temperature, there is plenty of leeway for this relatively conventional neuromuscular system to move the wings during flight.

Measurement of wing beat frequency and muscle potentials in several insects was undertaken in an attempt to correlate these contrasting observations, and to find out more, if possible, regarding the novel mechanism described by Pringle.

[1] The work described in this paper was done under a contract between the Medical Division, Chemical Corps, U. S. Army, and Tufts College. Under the terms of this contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

Method

The insect was narcotized with carbon dioxide and two small holes were made in the thoracic tergum. A glass stylus bearing two fine electrodes of 36 gauge platinum wire was cemented to the tergum with paraffin wax, in such a way that the electrode tips passed through the holes to lie among the thoracic muscles (Fig. 1). The stylus was placed in the needle holder of a crystal phonograph pick-up which was connected to the input of a conventional biological amplifier through 10 megohms. The electrodes were connected to another amplifier through loops or mercury cups. The movements of the thorax and the potentials occurring within were recorded simultaneously on a double-beam cathode-ray oscillograph.

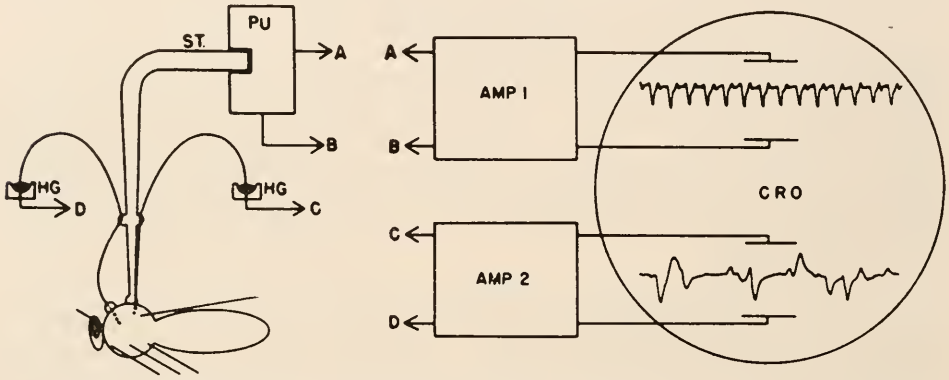

Figure 1. Apparatus used to make simultaneous records of thoracic movements and spikes during stationary flight. Platinum electrodes are fused to a glass stylus (ST) and make contact (CD) through mercury cups (HG) with amplifier. The stylus is inserted in a crystal phonograph pick-up (PU) which is connected (AB) with another amplifier. Removable platform below tarsi is not shown.

Stationary flight was initiated by the removal of a platform upon which the insect stood, and was maintained in some cases by a stream of air directed towards the head of the insect.

Thoracic Movements and Potentials During Stationary Flight

Periplaneta. Male roaches flew well at temperatures above 22° C. The wingbeat frequency lay between 25 and 30 per second. The relation of muscle spikes to wing movements was about as expected on the basis of the muscle studies. In records (Fig. 2 C) made with electrodes in various groups of thoracic muscles, a compound spike potential recurred at the same frequency and in fixed phase with the cycles of thoracic movement during flight. The record of the latter is quite complex, showing many harmonics, although the fundamental frequency was found to correspond with the cycle of wing elevation and depression, by viewing the insects simultaneously with stroboscopic light.

Agrotis. Several specimens of this moth were flown in the same manner. This insect showed slightly higher wingbeat frequencies (35 to 40 per second at room temperature) although the spike potential recorded from thoracic electrodes maintained the same 1 : 1 relation with the cycle of wing movement (Fig. 2 A).

At the onset of flight, the first spike was always followed by a movement, and during steady flight the spike appeared to be compounded of activity in several units. The spontaneous termination of flight took the form of a series of stepwise decreases in the amplitude of thoracic movement, accompanied by corresponding stepwise decreases in the height of the spike potential.

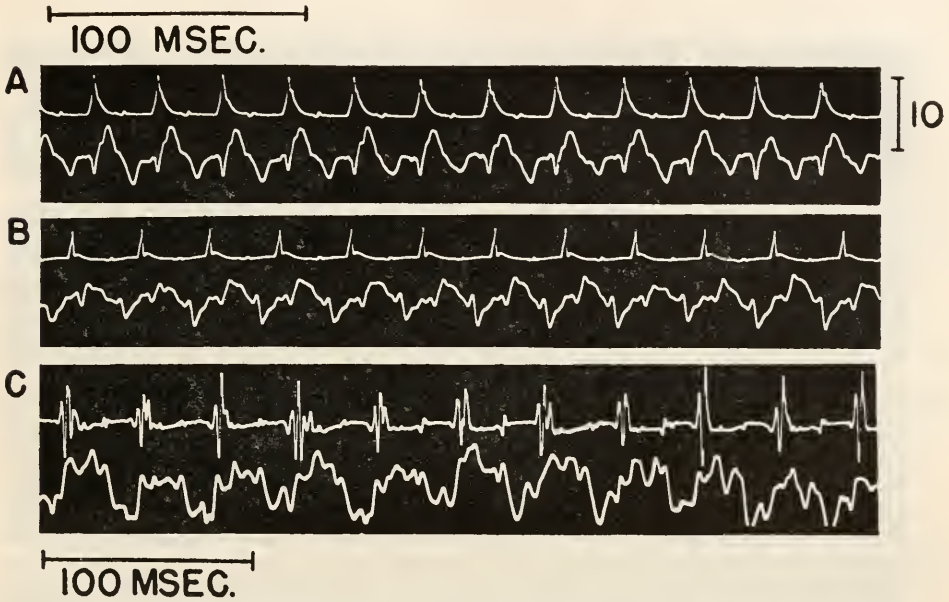

FIGURE 2. Spike potentials (upper trace) and thoracic movements (lower trace) in Agrotis with wings (A) and without wings (B), and Periplaneta with wings (C). Upper horizontal calibration applies to A and B, and lower to C. Vertical calibration, 10 millivolts.

Diptera. Flies belonging to the genera Calliphora, Lucilia, and Eristalis were flown in the same manner. The pattern of activity at the onset of flight is shown in Figure 3 A. The initial downward deflection is caused by the downward movement of the fly as the platform descends under its tarsi. The oscillation in the valley which follows indicates the moment when the tarsi lose their grip, following which the fly is flicked upward by the elasticity of the stylus. Flight begins about 60 milliseconds (flight reaction time) after contact with the platform is lost, and takes the form of a complex thoracic oscillation of steadily increasing amplitude (see also Fig. 3 D). No attempt has been made to analyze the pattern of thoracic movement during flight, although several characteristics may be noted: a) a fundamental frequency which corresponds with the frequency of wingbeat (this was checked by simultaneous stroboscopic observation); b) several harmonics, possibly produced by abrupt development of tension in different groups of flight muscles; 3) complex changes in the harmonic content and amplitude of the fundamental wave, which were particularly noticeable in Calliphora and Lucilia, and may be related to the characteristically erratic free flight of these insects. In Eristalis (Fig. 3 D), thoracic movement shows fewer fluctuations. The pattern of thoracic movement in these flies is very similar to the oscillogram

of the sound produced by Drosophila during flight (Williams and Galambos, 1950), and further harmonic analysis of both sound and movement patterns along the lines indicated by these authors would be of value.

In contrast to these changes in wave form and amplitude, the frequency of thoracic movement remains fairly constant in individual insects at constant temperature. Many of the insects would take only short flights, ceasing activity

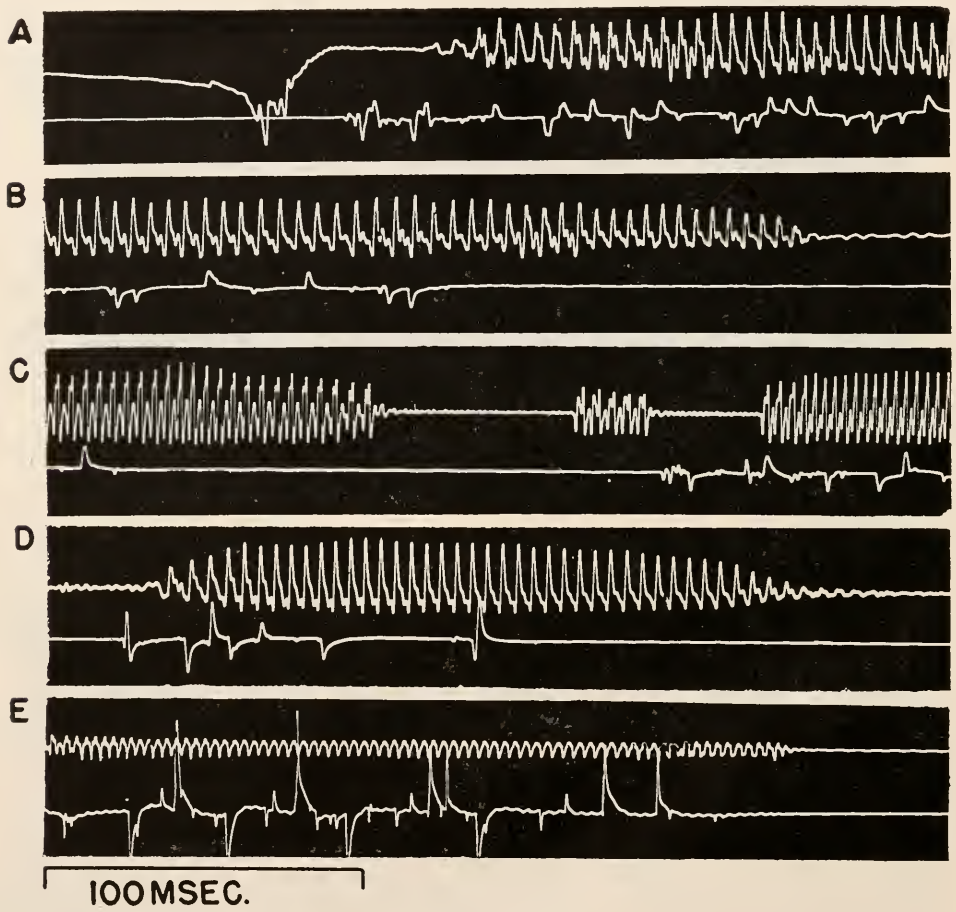

FIGURE 3. Spikes (lower trace) and thoracic movements (upper trace) in several flies. A. The onset of flight in Calliphora. B. Spontaneous termination of flight in the same insect. C. Irregular flight in the same insect following amputation of the wings. D. Short spontaneous (mid-air) burst of flight movements in Eristalis. E. Termination of flight in Lucilia after wing amputation. The spikes have been recorded with increased amplification to show the small sinusoidal potential.

spontaneously while suspended in mid-air. When this occurred (Fig. 3 B, D), the frequency of thoracic movement often remained unchanged while the amplitude declined steadily in stepless fashion, sometimes leaving a small trail of oscillation after the fly had apparently folded its wings. The smooth nature of the ampli-

tude change is particularly evident in the short flight of Eristalis (Fig. 3 D). Occasionally flight termination was quite abrupt (Fig. 3 C).

During the take-off, a series of spike potentials precede thoracic movements by 10 to 30 milliseconds (Fig. 3 A, D), and continue in an irregular fashion throughout the flight. Although at first the spike sequence appears to be random, once steady flight is established, a rough rhythm (Fig. 4) can be distinguished which has no phase-relation to the wingbeat. In long records it is possible to identify five or six sets of spikes, each set having its own sign, form, and repetition rate. In Figure 4 A and B, the downward spike shows constant form and size, and appears at regular intervals which differ from the intervals between similarly identifiable sets of spikes of opposite sign. Each set of spikes suggests activity of a single unit which is operating without synchronization with other units or with the sequence of muscular changes. In short or abortive flights (Fig. 3 D), a single short burst of spikes in several units may precede the peak of thoracic movement.

Each spike appears to have a duration of 5–10 milliseconds and a magnitude of 5 to 20 millivolts. In this respect, it is quite similar to the muscle potential recorded in Periplaneta (Roeder and Weiant, 1950). Increased amplification reveals in some (Figure 3 E) but not all records of thoracic potentials a low-voltage sinusoidal wave previously noted by Pringle (1949). This low-voltage wave follows the thoracic movements in frequency and amplitude, and may be an artifact due to the movement of electrically polarized muscle under the electrode pair. However, its origin is uncertain and it is discussed later.

Upon termination of the flight in mid-air (Fig. 3 B, D), thoracic movements continue for 20 to 40 cycles after cessation of the spike sequence. Although spikes were never detected when the fly had been motionless for some time, in one case (Fig. 3 C), following wing amputation an insect showed quite erratic bursts of activity. During one of these bursts movement ceased abruptly at the usual interval after the cessation of spike activity. One-tenth of a second later there was a short burst of five cycles of thoracic movement without the usual introductory spikes. After another pause spikes resumed followed by the return of steady flight.

Hymenoptera. Several specimens of the wasp, Vespa, were flown in a similar manner. Flight was steady and well sustained, and a regular sequence of thoracic movements and spikes bore, as in the flies, no phase relation with each other (Fig. 4 C). On the whole, the spikes in the wasp showed a more pronounced rhythm of their own, although the same mechanism appears to be operating in both flies and wasp. Several unsuccessful attempts were made to fly bees, but they showed a high mortality following insertion of the electrodes.

Loading, Wingbeat Frequency, and Spike Frequency

From the incomplete survey reported above, it is apparent that the relatively low wingbeat frequency (20 to 40 per second) occurring in the roach and moth is associated with spikes recurring once during each wingbeat. On the other hand, the high wingbeat frequency (over 100 per second) recorded in the flies and wasp is associated with an asynchronous sequence of spikes at much lower frequency. In order to determine whether there is a basic difference in the mechanism of muscle excitation in these two groups, or whether the difference is only quantitative, de-

pending upon the wingbeat frequency, attempts were made to alter the loading of the wings.

The mass of air moved by the wing has a damping effect upon the wingbeat, reducing its frequency. This relation has been subjected to rigorous analysis by Chadwick and Williams (1949) for Drosophila, in which amputation of the wings or flight under reduced atmospheric pressure increases the wingbeat frequency as much as 100 per cent. Therefore, attempts were made to alter the wingbeat frequency of the insects mentioned above by amputation of the wings close to the thorax. This operation appeared to have little or no effect upon the ability of the insects to "fly" when attached to the stylus.

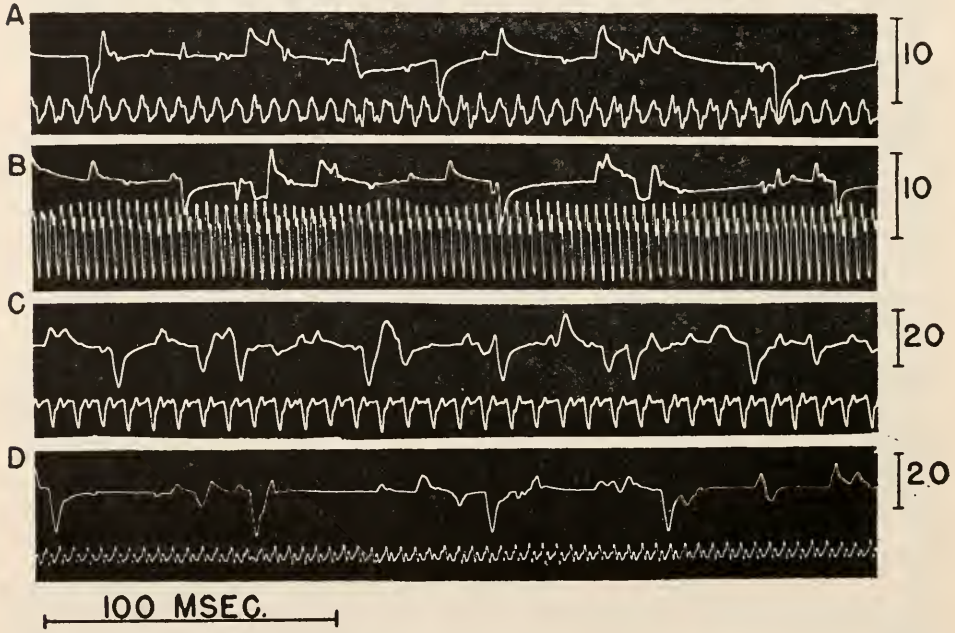

FIGURE 4. Spikes (upper trace) and thoracic movements (lower trace) during steady flight. A. Lucilia. B. The same after amputation of the wings. C. Vespa. D. The same insect after amputation of the wings. Vertical calibration, millivolts.

In the roach and moth (Fig. 2 B), close amputation of the wings appears to have little effect on the wingbeat frequency. In the latter insect there is a small but consistent decrease in frequency (Table I) although perfect synchrony between spikes and wing cycles is maintained. This suggests that the motor mechanism of the wings in these insects is driven by a central nervous pacemaker whose activity is more or less independent of the load. The slight but constant decrease in frequency in the moth could be due to the elimination of sensory endings of stimulatory function located on the wings.

In the flies (Figs. 3 C, 4 A and B) and wasp (Fig. 4 C and D), amputation of the wings produced the expected increase in the frequency of thoracic movement. While the frequency of thoracic movement almost doubled after this operation, the sequence of spikes recorded from the thoracic muscles either showed little change

or declined in frequency. Changes in wingbeat and in spike frequency following wing amputation are summarized in Table I. It will be noted that while the wingbeat spike quotient remains at unity in the roach and moth, it increases from a value of 4–18 when the wings are intact to 14–40 after wing amputation in the flies and wasp. The increase in w/s quotient is due both to an increase in wingbeat frequency and to a decrease in spike frequency following wing amputation.

TABLE I

Frequency of thoracic vibration and potentials during flight *

Temp. °C.	Insect	With wings			Without wings		
		Wing rate/sec.	Spikes per sec.	Quot. W/S	Wing rate/sec.	Spikes per sec.	Quot. W/S
24	Periplaneta	28	28	1	33	33	1
22	Agrotis	30	30	1	26	26	1
24	Agrotis	41	41	1	39	39	1
		39	39	1	37	37	1
		42	42	1	40	40	1
24	Vespa	119	22	5	203	13	16
		112	29	4	201	14	14
25	Calliphora	138	11	13	186	8	22
		146	16	9	193	8	24
26	Lucilia	156	10	16	286	8	36
		140	8	18	285	7	41

* Measurement of spike frequency could be made only when this had become constant after several minutes of steady flight. This condition was not attained with all of the insects flown. Measurements were made to the nearest whole number.

Thus, the neuromuscular mechanism of the flies and wasp appears to be once more in contrast with that of the roach and moth. The increase in wingbeat frequency following reduction of the wing load might be considered to be due to a reduction in the natural period of the mechanically oscillating system envisaged by Pringle. On the other hand, the spike frequency in both groups shows either no change or a definite decrease following removal of the wings. This could be due either to a reduction in sensory input to the nerve centers regulating flight (due to elimination of sense organs on the wings), or in the case of the flies and wasp, to a central nervous mechanism regulated by negative feed-back from proprioceptors sensitive to the excursion of wing-base, thoracic sclerites, or indirect flight muscles.

ELECTRICAL STIMULATION OF THE FLIGHT MUSCLES

Indirect electrical stimulation of the muscles of the leg (Pringle, 1939), or of the exposed tergal remotor muscle (Roeder and Weiant, 1950) of the roach produces twitches always preceded by a muscle spike potential. In contrast, Pringle (1949) noted that the indirect flight muscles exposed by hemisection of the thorax

of a fly could not be excited by any form of electrical stimulus applied either to the ganglion or to the muscle surface, while the muscles of the legs and proboscis could readily be made to contract by electrical stimulation. This observation was confirmed on several genera of flies.

In the course of the observations reported above, many of the flies tested failed to fly upon removal of the platform, although they continued to move their legs. It is presumed that these failures were due to injury incurred upon insertion of the electrodes into the thorax. In several cases, electrical stimuli were delivered to these refractory flies via the electrodes normally used to record potential changes within the thorax. It was found that many of these insects could be made to "fly" continuously at their normal wingbeat frequency of over 100 per second, if stimulated with brief shocks at any frequency from 3 to 20 per second. This would occur only if the tarsi were out of contact with the platform. Stimulus frequencies less than 3 per second produced only a brief twitch of the wings, while frequencies higher than 20 per second produced no additional effect beyond flight movements at the normal flight frequency. One insect, a large specimen of Tabanus, could not be induced to fly even by this treatment. If the tarsi of this insect were in contact with the platform, stimulation produced a maximum extension of all legs. If the insect was suspended in mid-air, each stimulus produced a strongly damped oscillation of the thorax at approximately the natural wingbeat frequency.

These results may be compared with those of Heidermanns (1931), who stimulated the flight muscles of a dragonfly by means of electrodes inserted into the thorax. In this insect (which has a wingbeat frequency in the neighborhood of 30 per second) a wing movement followed each shock up to a frequency of 25–30 per second. At higher frequencies (45–50 per second) of stimulation, the muscles went into a tetanus.

Discussion

In considering these experiments, it should be recognized that the flight performance of insects prevented from making forward progress must differ in many details from that occurring during free flight. One has only to watch the maneuvers of a free-flying insect to realize that its performance must depend upon a number of delicately balanced sensory feed-backs (Pringle, 1948; Weis-Fogh, 1950) and muscular adjustments. For instance, in performing the experiments described above, it was noted that flies would take off more readily and fly for longer periods if the platform under the tarsi were allowed to rotate freely so as to simulate forward locomotion before the take-off. Again, the flight reaction time of 40–60 milliseconds (Fig. 3 A) is both variable and long compared with that (21 milliseconds) determined by high-speed photography of the movements of a free fly when a bullet jerks away the platform upon which it is standing (Herget, 1950).

However, the experiments completely confirm the observation of Pringle (1949) of the lack of synchrony between spikes and wing movement in flies, and indicate that a similar state of affairs exists in wasps and possibly in other Hymenoptera. On the other hand, roaches and moths which have a low wingbeat frequency appear to have a more conventional mode of excitation of the flight muscles.

Excitation of the Flight Muscles in Diptera and Hymenoptera

According to Pringle, the situation in flies could be explained on the assumption that the arrival of a motor nerve impulse (signalled by a spike) "alters the state of the contractile elements in such a way that they become susceptible to the stimulus of stretching. On being stretched, the myofibrils respond with a twitch-like contraction whose duration is controlled by the loading, and then relax." Alternate twitches in vertical and horizontal indirect flight muscles would thus maintain in a state of oscillation at its natural period the mechanically resonant system consisting of the muscle tension, elastic characteristics of thorax, and wing load. The presence of a nervously conditioned, mechanically resonant system is confirmed by many of the observations made in this paper, namely: a) constancy of wingbeat frequency during amplitude changes at the onset and termination of flight; b) smooth changes in amplitude under the same conditions; c) inverse relation between spike frequency and wing beat frequency upon changes in loading; d) increase in wingbeat frequency upon reduction in loading; e) lack of excitability in flight muscles when the thorax is opened and the mechanical system presumably disturbed; and f) possibility of maintaining morphologically intact but moribund flies in flight at their normal frequency by electrical stimulation at any frequency.

Although Pringle's hypothesis provides an adequate explanation of the operation of the wings of flies during flight, it appears to provide no way in which the first contraction can be initiated if stretch is the immediate cause for contraction. On the other hand, the arrival of a motor impulse cannot be the immediate excitatory agent, since spikes may appear many milliseconds before signs of muscular activity (Fig. 3 A and D) and recur at no fixed point during the cycle of wing movement. Therefore, the following concept is presented as an elaboration of Pringle's original theory. In order to bring the indirect flight muscles of flies to a point where they drive the resonant system outlined above, two factors are necessary. One factor is the resting tension of the muscles, which depends upon their attachment to the inner surface of the semi-rigid, roughly spherical thorax. The tension is presumably constant in a resting fly, but is substantially reduced by any operation which interferes with the mechanical continuity of the thorax, such as hemisection. The second factor will be termed the neural factor, since it depends upon the arrival of motor impulses. It is presumably low or absent in the resting intact fly but is built up in a series of increments and maintained by the arrival of nerve impulses as indicated by the recorded spikes. The build-up of the neural factor superimposed upon the pre-existing tension factor steadily raises the muscle excitability until a twitch occurs in one set of muscles. The twitch causes a sudden increase in the tension of the antagonistic group of muscles which have been brought to some point just short of threshold by impulses in another nerve fiber. This increment in tension carries them over threshold so that they shorten and increase the tension in the other group. At this point, the myogenic mechanically resonant system of Pringle takes over, the oscillation continuing so long as the injection of occasional impulses maintains the neural factor at a level sufficient to offset the damping effects of the load.

It must be supposed that both factors must reach a certain level before muscular activity commences. In the resting or moribund fly, the tension factor is present but the neural factor is so low that no activity occurs. In the hemisected

thorax, electrical stimulation may raise the neural factor, but the tension factor is reduced so that again no contraction occurs. The time course of the neural factor can only be approximated, since it is not accompanied by an electrical sign. It appears to accumulate and dissipate at a much slower rate than the time course of individual spikes, since the latter may precede muscular movement by 5–30 milliseconds at the onset of flight, and cease an even longer time before cessation of movement at flight termination. In a very brief flight the burst of spikes may be terminated even before thoracic movements reach their full amplitude. In some records, periodic increases in amplitude of thoracic movements appear to bear some relation to increase in the spike frequency. Thus, the change produced by the spikes develops and dissipates relatively slowly compared with the electrical time course of a spike, and the latter have a cumulative action in bringing about its development. Since five or six sets of spikes in the same record are independent of each other in frequency, the rate of accumulation and dissipation of the neural factor must vary in different motor units.

The importance of tension as a factor in the excitation and contractility of smooth muscle is widely recognized. Sudden distension of the ureter may cause either a single, or an oscillatory contraction of its wall (Bozler, 1947). In this case, the tension-initiated contraction is accompanied by a potential change which is not evident during contraction of the indirect flight muscles of flies, although the small oscillatory potential (Fig. 3 E) which follows the thoracic movements remains unaccounted for. Direct studies of the effects of applied tension on the excitability of insect flight muscle have yet to be made.

Types of Flight Muscle Excitation Compared

Since there is no information regarding the relation between spike and wingbeat frequency in insects belonging to other orders, one can only speculate upon the significance of the difference between roach and moth on one hand, and flies and wasp on the other. Perhaps the mechanical characteristics of the thorax of the flies and wasp favor a degree of muscle tension which makes this the dominant factor in muscle excitation after the system has been 'cranked up' by the neural factor. The system then operates at its natural period of vibration. By the same token, the less compact thorax and larger wing area of the roach and moth may not provide the requisite muscle tension and resonance necessary for myogenic oscillation; hence, the dominant factor in causing muscle excitation is the arrival of a motor nerve impulse.

A transition is suggested by the observations of Boettiger and Furshpan (1950 and personal communication). They consider the energy of the contracting flight muscles to be stored in a mechanical stop (click mechanism) which, when overcome by increasing muscle tension, causes an abrupt movement of the wings. Boettiger recorded potentials and movements in several flies, including a crane fly. This slow-flying, large-winged fly had a wingbeat frequency of 36 per second and a sequence of spikes which showed a general 1 : 1 relation with the cycle of wing movement. However, the spikes and wing movements were not locked precisely in phase as in the roach and moth. Amputation of the wings of the crane fly increased the frequency of thoracic movement to 90 per second, while the spike frequency was unchanged. Thus, the difference observed in the two groups of insects

may depend solely upon the physical characteristics of the system. Voskresenskaya (1947) recorded the response of the flight muscles of a locust when the ganglion was stimulated at various frequencies up to 100 per second. She noted a tendency of the muscles to contract at 18 per second and a sequence of flight movements after stimulation had ceased. Since this after-effect was blocked by nicotine, it was apparently due to a central ganglionic after-discharge, and not a myogenic oscillation similar to that observed in flies.

The data summarized in Table I also suggest a compromise between the two types of neuromusclar mechanism. Although wing amputation brings about an increase in wingbeat frequency in the flies and wasp, and either little or no change or a decrease in the roach and moth, this operation affects the spike frequency in the same way in both groups of insects. This suggests that during flight the motor output of the central nervous system of both groups depends in part either upon a sensory inflow from mechanoreceptors on the wings, or upon negative feed-back from receptors sensitive to the rate of deformation of the moving parts of the thorax, flight muscles or wingbase. The halteres of flies (Pringle, 1948) and hair plates on the head capsule of locust (Weis-Fogh, 1950) have been shown to regulate orientation during flight (see also Waterman, 1950), and may, together with the proprioceptors mentioned above, form part of the general sensory field which maintains the output of motor impulses to the flight muscles. Therefore, the neural component of the flight mechanism appears to be similar in both groups of insects, and the difference between them may lie merely in the greater importance of resting tension as an excitatory factor of the flight muscle in flies.

Summary

1. Simultaneous records were made of thoracic movements and potential changes within the thorax of a number of insects during stationary flight.

2. The American roach and a moth with a wingbeat frequency in the range 20–40 per second showed complete synchrony between spikes and movements, and little change or a decrease in the frequency of both phenomena when the wings were amputated.

3. Several flies and a wasp with a wingbeat frequency above 100 per second showed no synchrony between spikes and movements, the former appearing once in every 5–20 wingbeats. Amputation caused either little change or a decrease in spike frequency, while the wingbeat frequency increased by nearly 100 per cent.

4. While the muscles of the former group retain their excitability to indirect electrical stimulation after dissection, the flight muscles of the latter are inexcitable following hemisection of the thorax. However, if the thorax is intact the indirect flight muscles of flies can be driven at the natural flight frequency by any stimulus frequency above 3 per second.

5. It is concluded that in the flies and wasp, the level of resting tension determines a degree of excitability in the flight muscles. This excitability is augmented by the accumulation of a neural factor brought about by the arrival of motor nerve impulses (spikes). Upon threshold being reached in one set of indirect flight muscles, they contract, and by increasing the tension factor in the antagonists, bring them rapidly to threshold. Flight continues as a myogenic oscillation at a natural frequency determined by muscle tension, elasticity of thorax,

and wing load, provided the neural factor is maintained at a certain level to offset damping.

6. In the roach and moth, the resting tension does not appear to be sufficient to give the system resonance or to contribute greatly to the muscle excitability. Therefore, the arrival of a motor impulse is the determining factor in contraction of the flight muscles.

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ELECTRON MICROSCOPE STUDIES ON THE NORMAL AND COLCHICINIZED MITOTIC FIGURES OF THE ONION ROOT TIP (ALLIUM CEPA) [1]

ALBERT W. SEDAR AND DONALD F. WILSON

Department of Zoology, and Radiation Research Laboratory, State University of Iowa, Iowa City

The question of the origin and structure of the achromatic figure in both animal and plant cells is still open to investigation. Various techniques have been brought to bear upon this problem. For a discussion, the monograph of Schrader (1944) should be consulted.

Using the electron microscope, Beams, Evans, Baker and van Breemen (1950), and Beams, Evans, van Breemen and Baker (1950) have studied the structure of the amphiaster in the whitefish blastula and in crayfish testis. In a similar manner, we have observed mitotic figures in root tip cells of *Allium cepa* in an attempt to contribute to knowledge of the structure of the anastral type of spindle, and the origin of the cell plate.

Material and Methods

Onion bulbs were sprouted in tap water at room temperature. Excised root tips were fixed in Randolph's chrome-acetic-formalin mixture for 12 hours, or in weak Flemming's fluid for 15 minutes. One-millimeter segments of the meristematic portion were embedded in either of two filtered paraffin mixtures. At first, a mixture of 70 per cent paraffin (m.p. 70° C.) and 30 per cent bleached beeswax was used. Later, a mixture of 50 per cent Tissuemat (m.p. 62° C.) and 50 per cent beeswax was found to be more satisfactory. In either case, the material was passed through three changes of the mixture, during a total period of 30 minutes. Blocks 2 mm. square were sectioned at approximately 0.5 micron after the method of Beams, Evans, Baker and van Breemen (1950). Ribbons so obtained were spread on distilled water at 45° C. and mounted on grids previously covered with a supporting celloidin film prepared from a 2 per cent solution of celloidin in amyl acetate. The grids were dried over phosphorus pentoxide after sections were mounted. The embedding mixture was then dissolved away in three changes of xylol, and all grids were stored in a desiccator until examined.

In studying the effect of colchicine, two concentrations, 2.5×10^{-4} molar and 2.5×10^{-3} molar, were used.[2] Rapidly growing roots, 0.5 to 1.0 cm. long, were

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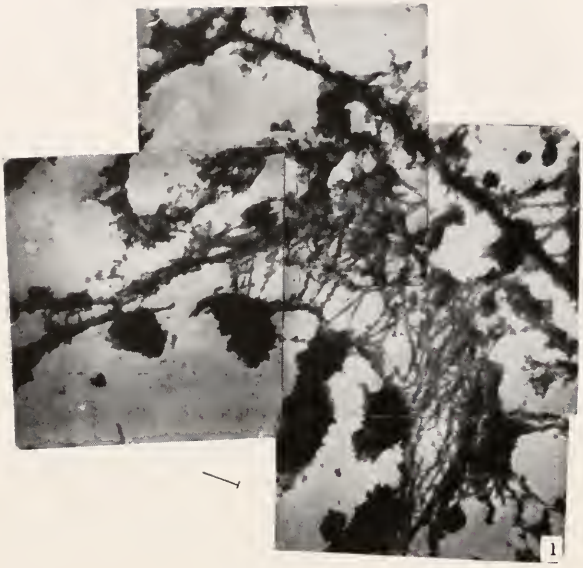
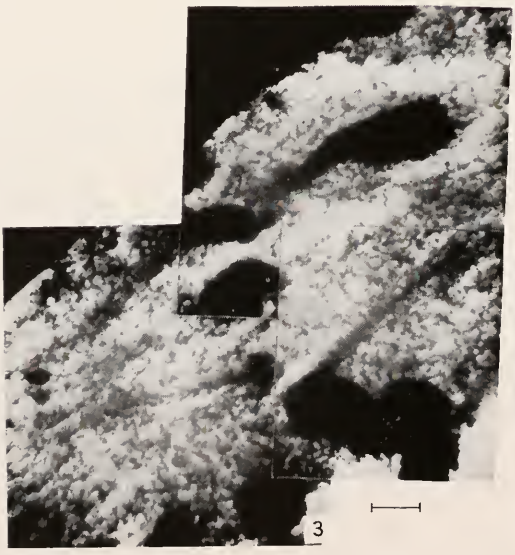
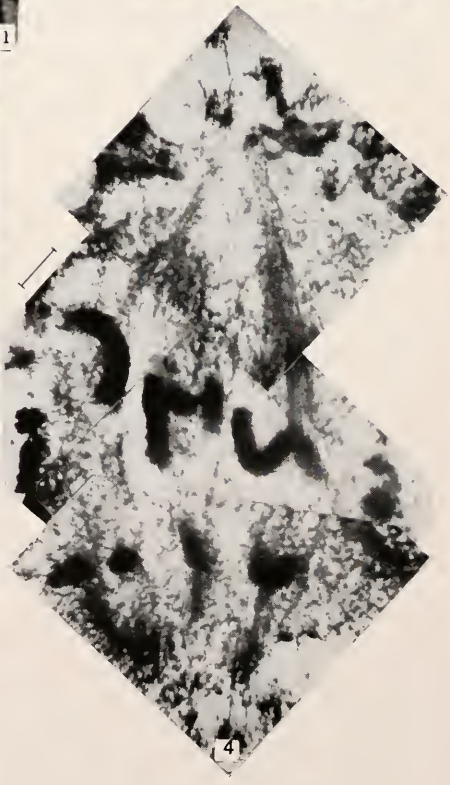
[2] The colchicine (U.S.P. XIII alkaloid) used was obtained from the Mallinckrodt Chemical Works.

PLATE I







exposed to these solutions for periods of 30, 45, 60, and 120 minutes. The root tips were then excised, fixed, and processed as described above.

Observations were made using a Model EMU–2B R.C.A. electron microscope equipped with an unbiased electron gun. A magnification scale representing one micron is drawn on each electron micrograph.

Results and Observations

Dividing Cells

1. *The Polar Cap:* Figure 1 is of a longitudinal section through half of a cell in prophase. A polar cap is evident, containing "spindle fibers" which are in contact with the nuclear membrane. In general, these fibers are oriented in the long axis of the cell and extend between the nuclear membrane and a dome-shaped structure which defines the outer margin of the polar cap. This border, in turn, is in contact with the lateral margins of the nuclear membrane.

The fibers are 600 to 800 Å. in diameter in these preparations, and exhibit a beaded structure, possibly due to fixation artifact.

Prior to the breakdown of the nuclear membrane these "spindle fibers" appear entirely extranuclear, and the nucleus contains no distinguishable spindle material.

2. *Metaphase:* Figures 2 and 3 are of longitudinal sections through the equatorial plate. Certain of the fibers seen are undoubtedly associated with the chromosomes, but details of the manner of their attachment are not revealed by this technique.

Chromosomal fibers are composed of smaller units oriented longitudinally to make up the main fiber. The diameter of these smaller units varies somewhat with the fixative used. In material fixed in chrome-acetic-formalin it is 500–800 Å., corresponding to the width of the individual "spindle fibers" of the polar cap. Fixation in weak Flemming's fluid results in fibers of somewhat smaller diameter.

3. *Anaphase:* The separation of homologous chromatids is shown in Figure 4. No interzonal fibers are to be seen.

A later stage is shown in Figure 5. The early cell plate is forming as a series of thickenings in or on the continuous fibers in the equatorial region. The spindle itself appears as a network of interconnecting fibers.

4. *Telophase:* Figure 6 is a later stage in cell plate formation. The cell plate has assumed a more definite structure and has already extended laterally to touch the cell wall on one side. The lateral margins of the spindle in the equatorial region are well defined and oriented. Elsewhere, the spindle material has become swollen and is disappearing. Reconstitution of the daughter nuclei is taking place. Areas of lesser density to the electron beam have appeared in the chromosomes.

Plate I

Figure 1. Prophase, showing polar cap containing "spindle fibers," and part of nucleus with nuclear membrane and chromosomes. Fixed in Randolph's CRAF solution.

Figure 2. Portion of metaphase plate showing chromosomes and spindle fibers. Fixed in Randolph's CRAF solution.

Figure 3. Portion of metaphase plate showing chromosomes and chromosomal fibers. Fixed in weak Flemming's fluid.

Figure 4. Portion of early anaphase showing chromosomes and chromosomal fibers. Fixed in weak Flemming's fluid.

Colchicinized Cells

Figure 7 shows what may be a very early manifestation of the action of colchicine on spindle fibers. The dose used here for a thirty minute exposure was 2.5×10^{-4} molar, considered by Levan (1938) to be in the threshold range for Allium. The chromosomal fibers appear to have lost their compactness by separation of the unit fibers.

Exposure to this same concentration for one hour produces the effect shown in Figure 8. The spindle material is seen to be slightly disoriented and fragmented. The chromosomes themselves appear to have lost much of their smooth contour.

Figure 9 represents a two hour exposure to this same colchicine concentration. The spindle material is swollen and badly fragmented. Much of it has disappeared. Some spindle or cytoplasmic material appears to be adhering to the chromosomes.

Figure 10 results from a 45 minute exposure to a concentration of 2.5×10^{-3} molar colchicine. In general, the effect obtained is comparable to the longer exposures to the more dilute solution.

Discussion

In a study of this kind, the factor of artifact induced by the method of preparation of the material for examination is necessarily present. For this reason, a certain amount of caution must be exercised in interpreting the electron micrographs. In general, however, our results agree with and in some cases extend the cytology as observed with the light microscope.

The fibrous nature of the chromosomal fibers, continuous fibers, and the "spindle fibers" of the polar cap is quite apparent in electron micrographs. The objection that this fibrous structure is a nonsignificant artifact is not considered to be valid today. In this connection, the review of Schrader (1944) should be consulted for details of other work.

With the electron microscope it is possible to demonstrate a compound structure of chromosomal fibers in onion. Beams, Evans, van Breemen and Baker (1950) have similarly shown this to be true in crayfish testis. However, Mottier (1903) had already reported that chromosomal fibers in Lilium were composed of "bundles of fibers" as the result of his observations with the light microscope. He did not observe a similar compound structure of continuous fibers, and we have not seen it in the onion.

In view of the widespread controversy which still exists concerning the production of artifacts in the mitotic spindle by fixation, we have employed two different types of fixatives in this study. The fibers appear of larger diameter when the chrome-acetic-formalin fixation is used than when a very weak Flemming's fluid is employed. However, the nature of the fibers in other respects is not seen to be altered. Preliminary results of a study of the effects of fixation in dilute osmic acid solutions and in osmic acid vapor indicate less swollen (or more contracted) chromosomes, more delicate spindle fibers and cytoplasm than with weak Flemming fixation. The same general character of these structures is, however, retained.

In the course of this work, several examples of a faint and rather ill-defined transverse banding of chromosomal fibers were seen. The phenomenon was ob-

PLATE II

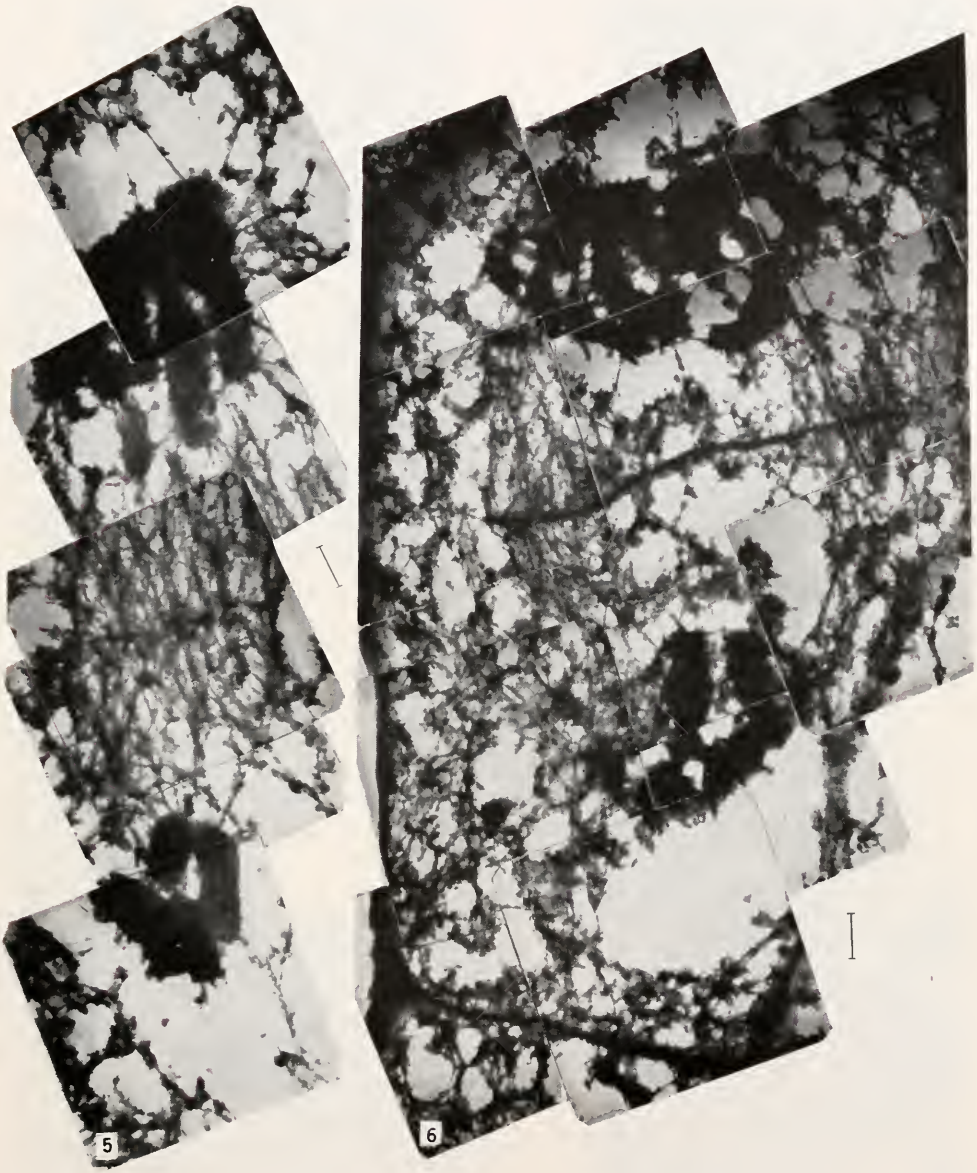
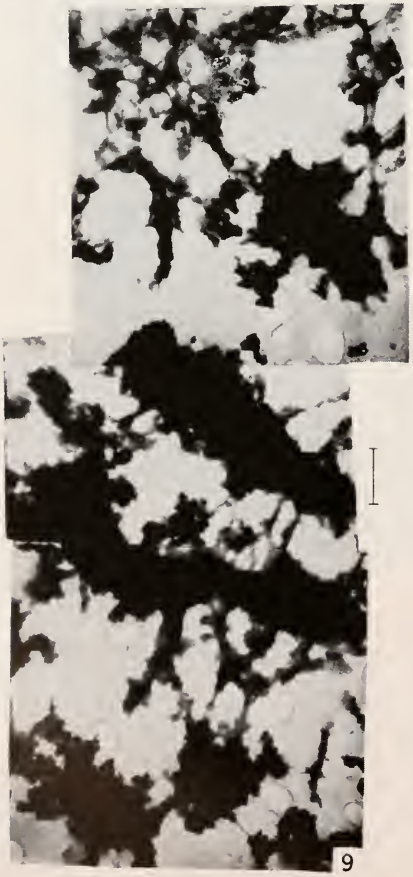
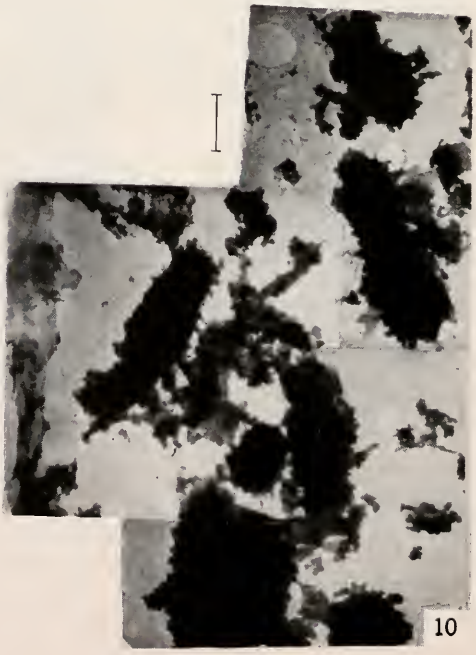


FIGURE 5. Late anaphase, showing spindle, early cell plate and daughter chromosomes. Fixed in Randolph's CRAF solution.

FIGURE 6. Telophase, showing reconstitution of daughter nuclei, phragmoplast, and cell plate extending to cell wall on one side. Fixed in Randolph's CRAF solution.

PLATE III





served in material fixed both in Randolph's and in Flemming's fluids. This banding was noted only in chromosomal fibers and only in metaphase or early anaphase. It does not reproduce well, although it can be seen in the original photographs. The banding is of a much finer nature and of smaller period than that described by Beams, Evans, van Breemen and Baker (1950) and is suggestive of a periodicity in the ultrastructure of the unit fibers. Such a periodicity has been predicted by several workers (see Beams, Evans, van Breemen and Baker 1950), but the phenomenon seen in our preparations cannot be considered anything more than suggestive at this time.

Robyns (1924, 1929) has described in detail the structure of the polar cap and its relation to spindle formation in both living and fixed preparations of onion and similar root tips. For a summary of earlier work, these papers should be consulted. Polar caps are described as clearly differentiated areas in the cytoplasm lying in contact with the nuclear membrane at the poles of the future spindle. In late prophase, these dome-shaped caps may extend as far as the equatorial region of the nucleus. Subsequent to the breakdown of the nuclear membrane, the polar caps enter into the formation of the spindle.

Our preparations show a definite fibrous structure in the polar cap prior to the breakdown of the nuclear membrane. Moreover, the outer margin of the polar cap is clearly seen to be delimited by a membrane-like structure. This membrane appears in electron micrographs to be of much the same structure as the nuclear membrane, and to fuse with it. However, it cannot be said that a definite splitting of the nuclear membrane has occurred in the formation of the polar cap.

The origin of the cell plate in higher plants has been described from fixed material as forming from a series of equatorial swellings in the spindle of cytokinesis (see Timberlake, 1900, for a summary of early work). Robyns (1929) disagrees with this view on the basis of his observations of living material. He concluded that the cell plate makes its appearance in the form of an undulating lamella, and believes that the lamella fragments under conditions of fixation into granules which appear attached to the spindle fibers. Becker (1938) stated that the cell plate first becomes evident as droplets exhibiting Brownian movement. These droplets form from the cytoplasm and eventually coalesce to form the cell plate. Although our preparations are fixed, the earliest indication of cell plate formation which we have observed is a series of thickenings of material continuous with, and of the same appearance as, the spindle fiber substance.

Levan (1938) has observed that the threshold concentration of colchicine which will produce an effect upon mitosis in Allium root tip cells lies in the range of 0.005 to 0.01 per cent with a four hour exposure. Levine (1943) has reported that with the 0.01 per cent concentration, the number of metaphases in Allium root

PLATE III

FIGURE 7. Portion of metaphase plate showing separation of chromosomal fibers resulting from 30 minutes exposure to 2.5×10^{-4} molar colchicine solution. CRAF fixation.

FIGURE 8. Disoriented fibers and eroded chromosomes resulting from 1 hour exposure to 2.5×10^{-4} molar colchicine solution. CRAF fixation.

FIGURE 9. Swollen and fragmented fibers resulting from 2 hours exposure to 2.5×10^{-4} molar colchicine solution. CRAF fixation.

FIGURE 10. Swollen and fragmented fibers resulting from 45 minutes exposure to 2.5×10^{-3} molar colchicine solution. CRAF fixation.

tips increases with increasing time of exposure to reach a maximum at about 24 hours. It is now well known that colchicine brings about the arrest of mitosis by destruction of the spindle. Since our purpose was to study the effects of colchicine upon the spindle fibers themselves, concentrations near the threshold and very short exposure times were chosen.

The very earliest effect which may have occurred was observed to be a spreading apart of the units which make up the compound chromosomal fiber. Longer exposures resulted in a loss of orientation of the spindle fiber material and a change in the normal contour of the chromosomes. Although chromosomes do not dissolve away in colchicine poisoning, Östergren (1944) has reported that colchicine causes a contraction of the chromosomes. Whether or not this has occurred in our preparations is not clear, but it does appear that some cytoplasmic or spindle material has adhered to the chromosomes, or else some of the matrix material has been eroded.

Longer exposure to the more dilute solution, or short exposures to the stronger of the two solutions resulted in swelling of the fibers and apparently a solubilization of their substance, since only fragments remain in the fixed preparations.

Beams and King (1938), using the ultracentrifuge, presented evidence that the effect of colchicine on wheat root tip cells is to lower the viscosity of the cytoplasm and to destroy or inhibit the gelation associated with spindle formation.

We have observed a swelling and fragmentation of spindle material in preparations of normal telophases in connection with the breakdown of the spindle. This swelling and dissolving of spindle material therefore appears to be the visible manifestation of both normal and colchicine-induced spindle breakdown, at least in our fixed preparations.

Summary

1. The electron microscope was used to study normal and colchicinized mitotic figures in onion root tip cells.

2. The polar cap contains "spindle fibers" prior to the breakdown of the nuclear membrane. It is bounded at one margin by the nuclear membrane and on the other by a membrane similar to and apparently continuous with the nuclear membrane. No spindle material is distinguishable inside the nucleus prior to the breakdown of the nuclear membrane.

3. Chromosomal fibers are composed of several smaller unit fibers oriented in a longitudinal fashion along the main axis of the fiber. This compound structure was not observed in continuous fibers.

4. Spindle fibers differ somewhat in diameter with different fixation. Flemming's fluid fixation results in a slightly smaller fiber diameter than that obtained with fixation in Randolph's CRAF mixture. The general character of the fibers in both preparations is essentially the same.

5. The origin of the cell plate is briefly discussed in the light of the work of early investigators. Electron micrographs show an origin by thickenings in the equatorial region of the spindle.

6. The effect of short exposures to near-threshold concentrations of colchicine is a progressive swelling and solubilization of fiber material.

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HOMOPLASTIC GRAFTING IN STENTOR COERULEUS

PAUL B. WEISZ

Arnold Biological Laboratory, Brown University, Providence, R. I.

Grafting techniques applied to the study of cell aggregates have long been among the most powerful tools of the experimental biologist. For technical reasons, similar procedures have not been generally applicable to single cellular units, but the desirability of such methods is evident.

Among ciliates, fusion complexes made up of two or more individuals are not new. In an important paper, Fauré-Fremiet (1945) reviews most of the known cases. He shows that such complexes either are found fortuitously in mass cultures, or can be produced by experimental or bacterial interference with the fission process, a procedure which leaves the daughter individuals joined together. Deliberate grafting has not yet been described, probably because physico-chemical peculiarities of the protoplasm in most ciliates generally preclude successful fusion of individuals by experimental techniques now available.

The feasibility of grafting in the heterotrich *Stentor coeruleus* has first been pointed out by Tartar (1941). He finds that "if two cells are cut and then immediately pressed together . . ., the two will fuse as one." Further exploration by the present writer has led to the development of a relatively simple method which permits the controlled, oriented fusion of two or more whole Stentors, or of specific fragments derived from several parent organisms.

Beyond the description of the methods employed, the aim of this paper is to set forth the broad principles which have been found to underlie grafting phenomena in *S. coeruleus*. Particular attention will be given to: the circumstances in which stable or unstable fusion complexes are formed, following the union of different portions of ectoplasm, endoplasm, and nuclei from two or more parent Stentors in several possible combinations and orientations; the reorganizational processes which can be observed in differently constituted fusion complexes; and the morphogenetic mechanisms which are operative in these phenomena.

Materials and Methods

Stentors were selected from cultures maintained in this laboratory for several years. The animals were fed intensively so that large specimens (up to 350 micra in diameter) were available for experimentation. Preliminary attempts at grafting had shown that any successful technique must permit (a) the maintenance of a desired orientation and contiguity of two whole animals or fragments for at least 30 to 40 seconds; and (b) the simultaneous rupturing of the apposed pellicles and ectoplasmic layers, so that the exposed endoplasms can flow out and fuse before the formation of surface precipitation membranes. The latter normally appear within 1 or 2 seconds.

The procedure finally adopted permitted the preparation of fusion complexes of any desired volume or specific constitution, and did not involve any loss of protoplasm during the operation. Several drops of a viscid suspension of methyl cellulose in water were spread on a glass slide, in a layer approximately 1 to 2 mm thick. A small drop of water containing two Stentors was pipetted into this layer. By manipulation with needles under a dissecting microscope, the animals were pushed gently into the methyl cellulose until they came to rest side by side. If the methyl cellulose had proper consistency, locomotion of the animals was prevented, and they could be maintained in a desired orientation and in continuous contact for as long as 1 minute or more. Sharp steel needles were used to rupture the apposed surfaces. When properly done, fusion was usually accomplished at the first attempt. If necessary, repeated ruptures could be made until the graft had taken. The fusion complex, along with a minimum of methyl cellulose, was transferred to culture water and the double organism was carefully freed from the embedding medium. After waiting 20 to 30 minutes for the fused organisms to heal firmly together, further operative steps could be undertaken. Doublets could be joined to yield multiple fusion complexes; specific differentiated organelles could be excised from one or both of the units; macronuclei of either of the components could be removed wholly or partially; or any combination of these procedures could be carried out. If small fragments were to be grafted to whole animals or to other fragments, two intact Stentors were first joined, and the required excisions were performed later.

Homopolar Doublets

The long axes of organisms constituting such doublets are oriented in the same direction and in the same sense. Categories 1 to 4, below, comprise experiments on parabiotic homopolar complexes, while category 5 deals with telobiotic homopolar doublets. In the former, the results are the same whether the units are joined in ventral-to-ventral, dorsal-to-dorsal, lateral-to-lateral, or any other combination. Analogously, in telobiotic doublets it is immaterial to what extent the units are rotated relative to each other. The following descriptions are based on a study of at least three cases in each type of experiment.

1. *Parabiotic homopolar fusion; no other experimental modification.* No visible changes occur for the first 4 to 6 hours. The two macronuclear chains are distinct and lie approximately parallel. Both peristomes and both holdfasts remain normal and functional, and both contractile vacuoles persist, maintaining, however, an independent rhythm of pulsation. Irritation of one member of the fused pair leads to smooth simultaneous contraction of both members, indicating an early functional integration of neural and contractile organelles.

Changes are first noticeable in the holdfasts. Approximately 5 to 6 hours after fusion, one of them decreases in size. If the components of the doublet are of unequal volume, the foot of the smaller member is invariably affected. The reduced foot later loses its adhesive properties, becomes permanently retracted, and is finally resorbed completely. Twelve to 18 hours after fusion, one of the contractile vacuoles and one of the oral regions (again the smaller structure, in each case) begin to dedifferentiate. In the oral area the gullet disappears first, and in the ensuing hours the entire peristome is gradually resorbed. Some 24 hours

after fusion, the original doublet has the appearance of a large single Stentor, although the two macronuclear chains are still distinct. Within a further 24-hour interval physiological reorganization occurs (*cf.* Weisz, 1949a), a process which results in the replacement of the persisting oral region by newly differentiated oral organelles, and in the transformation of the macronuclei into a single chain composed of proportionately larger nodes. A normal single individual is thus reconstituted. Vegetative division generally takes place within a short time.

In its essential features, this reorganizational sequence is identical with that described by Balbiani (1891), Johnson (1893), and Weisz (1951) for doublets of *S. coeruleus* found in mass cultures, or produced experimentally by interference with the process of fission. The sequence differs fundamentally from that in certain homopolar doublets of a variety of ciliates, *e.g., Urostyla weissei* (Fauré-Fremiet, 1945), or *Euplotes patella* (Kimball, 1941), in which the double organization is stable, and may give rise to two doublets by fission.

2. *Parabiotic homopolar fusion; excision of one or both of the systems of oral organelles, or one or both of the holdfasts.* If one of the oral regions is removed, regeneration does not occur. This holds true regardless of the initial relative size of the region. The foot of the gullet-less member is resorbed some 6 hours after fusion, while the contractile vacuole persists for 12 hours or more. Complete reorganization to a single individual, including the formation of a single macronuclear chain, is accomplished 24 hours after the operation. In effect, experimental removal of one oral region leads to a process of resorption similar to that described in No. 1, except that it is more rapid. Suppression of the gulletless member is always involved, regardless of its initial relative volume.

Analogous results are obtained if one of the holdfasts is excised. Regenerative replacement never occurs, and the persisting foot becomes the definitive holdfast of the reorganized single individual.

If both oral regions are cut off, only a single region ever regenerates. Using surface peculiarities and macronuclear configurations as identifying criteria, it has been found that the regenerating region is always part of the larger member of the fused pair. In the non-regenerating unit the foot, and later the contractile vacuole, are resorbed. As above, a single individual is reconstituted within 24 hours after fusion. Results are analogous if both holdfasts are removed, *i.e.*, only a single foot regenerates.

3. *Parabiotic homopolar fusion; complete removal of one macronucleus.*

a. Two whole animals are joined and all macronuclear nodes of one member are excised one by one; all except two or three nodes of the other member are similarly excised. (Enucleation procedures disrupt the constitutents of the endoplasm considerably. After one or two nodes have been excised, it is virtually impossible to ascertain the original location of most of the remaining nodes. Fortunately, two or three nodes just underneath the peristome field usually are not as easily dislodged as others. By removing all but these in one member, and all nodes in the other member, it is possible to prepare a system in which all macronuclear substance reliably derives from only one of the members. It is already known (Weisz, 1949b) that even a single anterior node is functionally equivalent to a whole chain of nodes, at any cycle stage.)

Doublets of this type ultimately reorganize into single individuals. The two or three nodes soon split up into smaller nodules. Each of these gradually in-

creases in size until a fairly normal macronuclear chain has re-formed. In other respects, the reorganization sequence parallels that observed in No. 1, and lasts approximately one to two days. In this reorganization, it is always the individuality of the enucleated unit which is suppressed.

b. A normal whole animal is joined with an animal undergoing physiological reorganization, at a stage when its nucleus is condensed maximally. The condensation nuclei never number more than three and are easily distinguished from the nodulated chain of the normal partner. Excision of these nuclear masses presents little difficulty, and at the same time the operation ensures that the entire nucleus, and only this nucleus, is removed. At the stage of maximal nuclear condensation, the oral region of the reorganizing member is only partially redifferentiated. The new peristome band is incompletely developed, and is not as yet fully aligned in its presumptive anterior position; a new gullet has not yet formed (*cf.* Weisz, 1949a).

Such doublets also reorganize into single individuals within one to two days. The incomplete oral region of the enucleated member never develops further, and begins to be resorbed after a few hours. In other respects the results are the same as in No. 3a.

4. *Parabiotic homopolar fusion: an animal undergoing physiological reorganization (condensation nuclei excised) joined to normal animal (oral region cut off).* Regenerative replacement of the excised oral region never occurs. On the other hand, the incomplete oral apparatus of the enucleated unit develops further and gives rise to a normally constituted peristome field and to a new gullet. These organelles become the definitive oral region of the single individual which is modelled from the components of the original doublet. Reorganization is usually complete after 24 hours.

5. *Telobiotic homopolar doublets.* Such doublets are produced by removing the foot of one organism and the oral region of another, and grafting the cut surfaces together. In effect, they represent large single individuals from the outset. Apart from the resorption of the contractile vacuole of the posterior unit, other reorganization phenomena cannot be observed. Experiments on regeneration and enucleation have been carried out. The results are wholly equivalent to those obtained in normal, single animals.

Heteropolar Doublets

The majority of experiments under this heading deal with telobiotic complexes, but parabiotic ones are also included. Telobiotic doublets in categories 6 to 9 are foot-to-foot heteropolar; they were produced by removing the holdfasts of two whole animals and grafting the cut surfaces together. Doublets in category 10 are mouth-to-mouth heteropolar. For each category, the results are the same, regardless of the relative degree of rotation of the units around their common axis. If this axis does not form a straight line from the outset, heteropolarity is never maintained. An originally obtuse angle between the components of the doublet becomes progressively more acute and, within three hours at the most, a homopolar parabiotic system has formed. Thus if necessary, varying amounts of lateral cytoplasm must be excised after fusion, until the doublet acquires a straight longitudinal axis. The following descriptions are based on a study of at least three cases in each type of experiment.

6. *Telobiotic (or parabiotic) heteropolar fusion; no other experimental changes.* Differentiated organelles do not undergo reorganizational changes, and both oral regions persist. Approximately 7 to 12 hours after fusion, a constriction becomes noticeable along the ectoplasmic suture line between the two components. The suture line is defined by the abrupt discontinuity and the derangement of the surface stripes where the components join. A similar suture is recognizable for many hours in homopolar grafts, but signs of constriction never appear in these. In heteropolar grafts, the constriction deepens progressively and 24 hours after fusion, only a slender strand may still connect the two members. Complete separation of the members occurs invariably, the points of last contact developing into new holdfasts.

In a given member, the number of macronuclear nodes at the time of constrictive separation is generally either greater or smaller than before fusion, although the total number of nodes in the doublet remains constant. Also, the volumes of the separated units may be strikingly different from their volumes before fusion, even allowing for the fact that small quantities of cytoplasm may have been excised during the grafting procedure. The constriction evidently cuts through the interior protoplasm regardless of whether nuclei and endoplasm in the constriction plane derive from one or the other component. It may be concluded that the plane of constriction is determined solely by the ectoplasmic suture line. The phenomenon of constructive separation is strikingly reminiscent of vegetative division, a process in which constriction takes place along a well-defined ectoplasmic fission line (Weisz, 1951).

A similar result has been described by Lund (1917) for heteropolar doublets of *Bursaria truncatella.* In this species, however, constrictive separation appears to occur only if the units are of equal volume; if they are not, "the smaller and weaker member sooner or later dedifferentiates and the whole or part of its substance becomes part of the stronger and larger member" (by redifferentiation with a reversed polarity). In *S. coeruleus,* on the other hand, constrictive separation takes place regardless of the initial relative volume of the units in the doublet, and none of the organelles ever dedifferentiate. (Moreover, reversal of polarity through de- and redifferentiation has never been observed in Stentor.) In other species studied, a heteropolar configuration is either remodelled into a homopolar one, or the doublet disintegrates within a short time (*cf.* Fauré-Fremiet, 1945).

7. *Telobiotic (or parabiotic) heteropolar fusion; removal of one or both oral regions.* In either case, normal regeneration occurs. Regeneration is completed long before the units separate. If the oral areas are excised only one to two hours before the units are expected to separate, they divide as fragments and regenerate thereafter.

8. *Telobiotic heteropolar fusion; excision of one macronucleus.*

a. Two whole animals are joined, and by methods described in No. 3a, all except 2 or 3 macronuclear nodes of one of the components are excised. Under these conditions, the heteropolar configuration is never maintained, even if all possible precautions are taken to produce an initially truly heteropolar doublet possessing a straight axis. Transformation into a homopolar parabiotic system ensues invariably within a few hours. Thereafter, the course of events is identical with that described in No. 3a.

If one of the units is not completely enucleated and even a single node is retained, heteropolarity persists and constrictive separation takes place.

b. A normal Stentor is joined with one undergoing physiological reorganization, and the condensation nuclei of the reorganizing member are excised. As in No. 8a, transformation into a homopolar doublet soon takes place. Thereafter, a single individual arises by processes described in No. 3b.

9. *Telobiotic heteropolar fusion; an organism undergoing physiological reorganization (condensation nuclei excised) joined to normal animal (oral region cut off).* A homopolar doublet soon forms as above. Subsequent reorganization follows the steps outlined in No. 4.

10. *Mouth-to-mouth heteropolar doublets.* Such complexes are produced by removing the oral regions of two whole animals and grafting the cut surfaces together. As in foot-to-foot heteropolar doublets, heteropolarity changes to homopolarity unless the doublet possesses a straight longitudinal axis.

Regenerative replacement of both oral regions begins four to five hours after fusion, the expected time interval at room temperature. Some 12 hours after fusion, regeneration is completed. Normal, well-formed peristomes and gullets have developed in each component. These oral organelles are situated laterally, along the suture line of the doublet. Constrictive separation does not take place. Twenty-four hours after fusion, the doublet has become remodelled into a homopolar parabiotic system, the two holdfasts now lying side by side, and the two oral regions occupying the anterior aspect of the doublet. Further reorganization into a single individual occurs as in No. 1.

Multiple Complexes

By grafting doublets to other doublets or to single animals, a considerable number of Stentors can be combined in any desired pattern. Complexes consisting of up to 6 individuals have been prepared, each complex representing a random or non-random pattern of homopolar or heteropolar grafts. The results may be summarized as follows.

a. In properly formed complexes, each component occupies a definite continuous space within the system. The space is initially identifiable by a virtually intact system of surface stripes, by a distinct chain of macronuclear nodes, and by normally functional oral organelles. Such complexes are viable, regardless of the orientation of the components.

The grafting procedure sometimes leads to a severe disruption of the surface organelles of the fusion complex. As a result, the spatial individuality of given units is destroyed more or less completely. This effect can also be achieved intentionally, by passing the operating needles rapidly and at random through the fusion complex. Such systems are not viable. Abortive attempts at surface reorganization are noticeable, but after some days vacuolation becomes increasingly apparent, and the complex ultimately disintegrates.

b. During the first 12 to 18 hours after fusion, more or less extensive ectoplasmic shifts and internal cyclosis-like rearrangements occur in viable complexes. Regardless of the initial fusion pattern, these shifts change the pattern into one of three characteristic types. In one of these, the system acquires a single major axis, and all units are aligned in parabiotic homopolar orientation. In a second

type, the complex is essentially biaxial. The two axes form a straight line, but have opposite sense, *i.e.*, the two oral poles are at opposite ends of the straight line, and the two pedal poles join at a common point, approximately mid-way along the line. Around each of these axes are grouped one or more units in parabiotic homopolar combination. These homopolar groups are consequently telobiotic and heteropolar to each other. In a third, less common, pattern of organization, the complex is triaxial. The three axes radiate out from a common (pedal) center and subtend roughly equal angles. One or more units are grouped around each of the axes in homopolar parabiotic combination.

The number of sub-units composing the complex does not influence the type of pattern emerging after 24 hours. Rather, a given pattern is partly an enhanced expression of a fundamental initial architecture of the complex, and partly a result of form-regulating rearrangements. The latter are in turn predictable from the spatial peculiarities of the fusion mass. Experiments indicate that the three patterns described are probably the only ones which can possibly emerge. Attempts to create radial tetraxial complexes always meet with failure. In such attempts, adjacent axes at best subtend 90-degree angles, which eventually increase to 180 degrees or to zero, bringing the units grouped around these axes into heteropolar or homopolar alignment. Thus, even if all four axes lie in one plane, the second pattern described above emerges. Random polyaxial and completely irregular complexes also transform into one of the organizations described.

c. A homopolar parabiotic group, whether it represents an entire complex or only a part, ultimately reorganizes to a single organism or sub-unit, by steps described in principle under experiment No. 1. Oral regions are dedifferentiated one by one, at a rate of approximately one every 24 hours. The last oral region persists.

Parabiotic homopolar groups which are telobiotic and heteropolar to each other separate by constriction after one or two days. This holds true for biaxial as well as for triaxial complexes. The latter, if not previously transformed into homopolar groups, may either split into three monaxial subcomplexes at roughly the same time, or may first give rise to one monaxial and one biaxial complex.

d. If one or more of the organisms which are originally grafted together are in late stages of the vegetative cycle, fission will occur while they are joined to the complex. Depending on the manner of fusion, the ectoplasm in the presumptive path of the fission line may either be partially excised or twisted out of position, or may lie entirely free and unobstructed. In the former case, fission cannot be completed (*cf.* Weisz, 1951). A new oral area and a (partial) posterior daughter individual are formed nevertheless, thus increasing by one the number of units in the complex. In the latter case, fission is completed and the anterior (or the posterior) daughter individual is constricted off from the fusion mass.

Discussion and Conclusions

The following general conclusions can be drawn from the data.

1. Grafting procedures in *S. coeruleus* yield fusion complexes which are invariably labile. A given complex, if viable, always reorganizes into one or more single individuals: (a) by breaking up into units through constriction, in which

case the constituent organisms of the complex are recovered more or less *in toto;* or (b) by stepwise dedifferentiation of all but one of the component units, in which case a single, enlarged but normal individual develops; or (c) by a combination of these two processes. Viability of the complex is contingent upon the retention of the essential individuality of each unit, particularly as regards the surface organelles. If the ectoplasm is too severely disrupted, the fusion mass ultimately disintegrates.

2. It is clear that the instability of fusion complexes cannot be due to either endoplasmic or nuclear incompatibility. This is substantiated both by the fact that grafting is possible at all, and by the observation that all homopolar and many heteropolar complexes give rise to individuals which contain nuclei and endoplasm from more than one source, in permanent, stable union. The lability of fusion complexes and the different experimental results must therefore be interpreted through ectoplasmic activities.

It has been shown elsewhere (Weisz, 1951) that in *S. coeruleus* all phases of morphogenesis decisively involve kinety I. According to Lwoff (1950) and his collaborators, this and every other kinety is a system of ectoplasmic organelles composed of a longitudinal row of granules, the kinetosomes, and a longitudinal fiber, the kinetodesma, which is situated to the right of the kinetosomes. Kineties are thus asymmetrical, being polarized both transversely and longitudinally. Since present experiments deal primarily with problems of morphogenesis, the results should be interpretable in terms of kinetal function.

3. Stepwise dedifferentiation of supernumerary units constitutes the method of reorganization in complexes composed of units which are initially or secondarily joined in homopolar and parabiotic orientation. In any homopolar association of organisms, all kineties are oriented in the same sense and in the same direction, *i.e.,* they are mutually homopolar themselves, both longitudinally and transversely. Thus, the net effect of this type of grafting is the interpolation, into an existing layer of kineties, of another section of ectoplasm containing identically oriented kineties. No fundamental change has occurred in kinetal orientation *per se.* The graft is integrated functionally and remains permanently in place.

On the other hand, the procedure introduces additional kineties I into the system, and an equal number of oral organelles, holdfasts, and contractile vacuoles. Why is stability and an equal status of "dominance" not retained by all of the sets of organelles and the kineties I? Indeed, an essentially identical question must be raised for any normal single Stentor: how are individual kineties prevented from exercising the same specialized functions as kinety I, particularly since it has been shown (Weisz, 1951) that all kineties are fundamentally equipotential? No fully conclusive answer can as yet be given to this important general problem. Ectoplasmic structures arise from, and are maintained by, specialized kinetosomes which in turn are derivatives of the kinetosomes in kinety I (*cf.* Weisz, 1951). It is clear, therefore, that all questions of physiological dominance involving differentiated organelles or kineties reduce to problems of kinetosomal dominance.

Present experiments and certain aspects of earlier observations on kinetal processes suggest a metabolic solution of the problem of kinetosomal dominance. If given kinetosomes were to possess, or could acquire, a competitive metabolic advantage over other kinetosomes, the morphogenetic potentials of the latter would remain or become inhibited. The experiments indicate that kinetosomes of the

largest sub-unit in a fusion complex generally acquire dominance over kinetosomes in other sub-units. This might imply that particular dominance relationships within the complex are due to quantitative differences of structure, and thus presumably of maintenance metabolism, among the sub-units. Dedifferentiation of the organelles in the smaller sub-units, and loss of specialized function of their kineties I, would then be a consequence of continuous successful competition by the kinetosomes of the sub-unit possessing a quantitative metabolic advantage.

Furthermore, kinetosomes of complexly differentiated organelles are dominant over kinetosomes of less complexly differentiated structures. In normal Stentors, for example, oral kinetosomes are dominant over those in kinety I: the latter obviously can not produce additional oral regions as long as one is already present, but can do so as soon as the oral kinetosomes are removed (Weisz, 1951). This relationship accounts for the observation (experiment No. 2, above) that in the absence of the larger oral region in a doublet, the smaller oral region becomes dominant over kinety I of the larger sub-unit, and regeneration of the excised structures cannot occur. In the absence of both oral regions, however, kinety I of the larger sub-unit becomes dominant over all other kineties, and this kinety alone gives rise to a new oral region. Analogous interrelationships hold true between kineties and holdfasts or contractile vacuoles, as the experiments indicate. In view of the fundamental equipotentiality of kinetosomes, dominance relationships in the above instances might again imply intrinsic, or micro-environmentally conditioned, metabolic specializations of kinetosomes. These specializations would determine the degree of complexity to which given organelles become differentiated.

4. In heteropolar complexes, the predominant method of reorganization entails constrictive separation (unless the system transforms secondarily into a homopolar complex). In these instances, kineties are oriented in the same direction, but in the opposite sense, *i.e.*, they are longitudinally and transversely heteropolar. Comparison with homopolar grafts indicates that the lability of these complexes is due primarily to the heteropolarity of the apposed kineties. As in other aspects of kinetal behavior (Weisz, 1951), properties of a magnet are suggested, inasmuch as like poles of the kineties appear to repel each other. Consequently, each unit in the complex should, and does, retain complete ectoplasmic individuality (even though an early neural and contractile integration is indicated). Dominance relationships are not established, and each unit therefore retains the essential characteristics of a free, single individual. This includes the capacity of regeneration, maintained by an independently functional kinety I (*cf.* experiment No. 7). Constriction itself, considered from the standpoint of a single unit, is a process equivalent to that following removal of the posterior region in a normal free animal: the region of the cut elongates and narrows down to a protruding tip, the latter developing into a new foot. As already noted, fissional constriction proceeds through very similar steps; the contrictional forces involved are probably the same.

5. The experiments in No. 3, No. 4, No. 8, and No. 9 show that macronuclei are cross-active from one unit to another. Nuclei in one unit not only maintain viability and normal function of organelles in an enucleated unit, but also support differentiation, *e.g.*, the completion of oral differentiation in the enucleated member in experiment No. 4. On the other hand, enucleation evidently does affect the status of dominance of a unit within a complex. Even though the enucleated unit

of a parabiotic doublet may be the larger component, the smaller nucleated member will nevertheless acquire dominance (*cf.* experiment No. 3a). Macronuclear cross-activity thus cannot fully compensate for the absence of nuclei in a unit. It is impossible to determine the reasons for this from the existing evidence, but since the question of dominance is involved, a solution in metabolic terms may again be indicated. Comparison of experiments No. 3b and No. 4 also shows that loss of the macronucleus does not affect the status of dominance as much as loss of the oral apparatus.

6. Secondary transformation of a heteropolar doublet into one which is parabiotic and homopolar occurs: whenever the two axes do not form a straight line; when one of the units is enucleated; and in mouth-to-mouth heteropolar associations.

Biaxial systems in which the two axes do not have the same direction are fundamentally monaxial from the outset. A major axis bisects the angle between the components and such systems are actually parabiotic, the area of fusion being confined to the most posterior regions. Subsequent reorganization merely makes the parabiosis more obvious and effects a more nearly homopolar orientation of the kineties.

An originally heteropolar doublet with an enucleated component represents a system in which the components are no longer co-equal. Despite macronuclear cross-activity, the enucleated unit loses an as yet indefinable characteristic of kinetal individuality (*cf.* preceding section), and a dominance relationship presumably arises within the complex. It is not apparent, however, how these conditions could lead to the initial change of kinetal polarity.

Transformation of mouth-to-mouth heteropolar doublets also remains unexplained. Such complexes maintain true heteropolarity until the two oral regions have fully regenerated. Only then does an orientational change occur. Present evidence does not show why constrictive separation does not take place instead.

Regardless of the specific conditions which induce orientational changes in given fusion complexes, the mechanism of transformation appears to be the same in every case. Unilateral resorption and a consequent shortening of kineties is probably involved.

7. While many of the results merely raise new problems, a sufficient body of evidence has accumulated to indicate that grafting phenomena in *S. coeruleus,* like fusion phenomena in other species (Fauré-Fremiet, 1945), are governed to a great extent by kinetal and kinetosomal processes. Kineties and kinetosomes should therefore hold clues to questions as yet unresolved. Among the most puzzling of such questions is the different behavior of fusion complexes in different species. Further work, conceivably supplemented by grafting techniques, may not only yield answers to this problem, but may also increase significantly our understanding of kinetal and kinetosomal function.

Summary

1. A technique is described which permits oriented grafting of two or more whole Stentors or fragments.

2. Fusion complexes in *S. coeruleus* are invariably labile. If the surface organelles of the component units are too severely disrupted, the complex disintegrates.

In viable homopolar systems, all but one of the units dedifferentiate, and a single enlarged individual is formed. In heteropolar systems, the units maintain their individuality and finally constrict apart, in a process strikingly reminiscent of fissional constriction. Random multiple complexes first acquire one of three characteristic patterns of organization, and then reorganize into single organisms by either or both of the methods mentioned.

3. In a homopolar parabiotic doublet, the unit which later dedifferentiates is always the smaller component. It cannot regenerate excised parts, even before its individuality is suppressed. On the other hand, if the oral organelles of the larger component are excised, they do not regenerate, and the smaller unit becomes dominant. In heteropolar complexes, each unit can regenerate excised parts, regardless of its initial relative volume.

4. Macronuclei are found to be cross-active, nodes of one unit maintaining normal function and the capacity of differentiation in an enucleated unit. However, the particular status of dominance of a unit is abolished by enucleation.

5. Questions raised by the experiments are shown to be reducible to problems of kinetal and kinetosomal function. As far as is possible, therefore, the results are interpreted in terms of activities of kineties and kinetosomes, ectoplasmic organelles known to be decisively involved in processes of ciliate morphogenesis.

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AN ANALYSIS OF THE DYNAMIC FACTORS RESPONSIBLE FOR THE PHENOMENON OF PIGMENT SUPPRESSION IN SALAMANDER LARVAE [1]

HARVEY EUGENE LEHMAN

School of Biological Sciences, Stanford University, and Department of Zoology, University of North Carolina, Chapel Hill, N. C.

Twitty and Bodenstein (1939 and 1944) and Twitty (1945) have shown that grafted urodele pigment cells which have a relatively rapid developmental rate are able to suppress the differentiation of more slowly developing pro-pigment cells. An examination of the correlation between differences in rates of development and degree of suppression (Lehman, 1950) has demonstrated that a relatively large, though not maximal, difference between the developmental rates of donor and host cells provides the most favorable condition for the suppression of pigmentation. The latter study also showed that although the inhibition of slowly developing cells may be virtually complete in pre-feeding and early larval stages, the effect is not permanent. The more slowly developing melanophores later appear in progressively greater numbers and make a substantial contribution to the final pigment pattern at metamorphosis. There is thus a clear indication that the phenomenon of pigment suppression of the type encountered here [2] should be considered a temporary condition associated with embryonic and early larval periods of pigment differentiation.

The experiments described in this paper are a continuation of the work cited (Lehman, 1950) and attempt to clarify further the developmental mechanics of the suppression phenomenon. To this end, three general aspects of pigment cell behavior have been investigated. Listed in the order treated, they are: A) chromatophore interactions influencing pigment cell migration, B) influences of environmental factors on chromatophore differentiation, and C) intrinsic species differences in pro-pigment cell behavior.

The writer gratefully acknowledges his indebtedness and appreciation to Professor V. C. Twitty, whose personal interest, encouragement, and critical suggestions played a large part in bringing this work to completion.

Materials and Methods

Embryos of five species of salamanders possessing different normal rates of development were used in the following experiments. Proceeding from the most

[1] Based in part upon data included in a dissertation submitted to the Faculty of Stanford University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Biological Sciences.

[2] Not to be confused with the type of pigment inhibition that results from the replacement of anterior trunk neural folds by anterior cranial folds (Niu, 1947). This type of pigment inhibition presents one of the most puzzling enigmas in the entire problem of pigmentation, and is entirely different in character from the suppression effect dealt with in this paper.

rapid to the slowest, this graded series consisted of *Amblystoma tigrinum, A. mexicanum* (the black axolotl), *A. punctatum, Triturus torosus* and *T. rivularis.* The differences in their developmental rates are graphically portrayed in an earlier paper (Lehman, 1950). The embryos of *T. rivularis* were obtained from Robinson creek near Ukiah, California, and those of *T. torosus* were collected from ponds and streams in the vicinity of Stanford University. The eggs of *A. punctatum* were shipped to Stanford University from Connecticut, North Carolina, and Illinois, and those of *A. tigrinum,* from Illinois. *A. mexicanum* eggs were obtained by temperature-induced spawning from axolotls maintained at the Stanford laboratory.

The experiments include the explantation, transplantation, and extirpation of neural folds and neural crest, and the transplantation of epidermis. Unless otherwise stated, experimental embryos along with appropriate controls were reared in individual dishes and kept at 14 to 18° C. The culture methods employed for *in vitro* studies followed the hanging drop technique described by Twitty (1945). The nature of the various graft and explant combinations, along with special details of methods and techniques, are given in conjunction with the description of each experimental series. Developmental age is given in terms of the Harrison stages for *A. punctatum* and *A. tigrinum* and the closely corresponding Twitty and Bodenstein stages for *T. torosus* and *T. rivularis* (see Hamburger, 1942, pp. 202–204, and Rugh, 1948, pp. 94–101). For brevity, single arrows have been used throughout the text to indicate the direction of donor-to-host graft combinations.

Experiments

A. Chromatophore interactions influencing pigment cell migration

Considerable experimental data (Twitty, 1944, 1945, and 1949, and Twitty and Niu, 1948) support the thesis that the primary motivating force in pigment cell migration is intrinsic to the chromatophore population itself, rather than being due to chemotactic (Rosin, 1943, and Holtfreter, 1947) or thigmotactic (Weiss, 1945, and Dalton, 1949 and 1950) responses to the tissue environments the cells may enter. The movement of pigment cells can be considered the expression of intercellular antagonisms (in the sense of Holtfreter's "negative tissue affinities" (1939)), which cause the cells to repel one another mutually and, in a manner of speaking, to strive for isolation. Twitty (1945) has shown that the same intercellular antagonisms that are responsible for migration also enable pigment cells to impede the migration of other melanophores that might attempt to invade an occupied terrain. The experiments in Series 1, 2, and 3 were carried out in order to discover the duration of the period in which pigment cells manifest mutual antagonisms.

Series 1. Duration of mutual antagonisms between chromatophores in vitro: Cultures of trunk neural crest and neural folds were prepared in the following manner. Mesoderm-free neural crest, along with a small amount of underlying neural tissue, was removed from Stage 23–25 tail bud embryos after the mid-dorsal epidermis had been carefully stripped away. Mesoderm-free trunk neural folds were taken from Stage 16–17 neurulae. The strips of excised neural crest and neural fold were divided into three parts of equal size and each piece was then isolated in a drop of Holtfreter solution on a cover glass. The cultures were sealed

PLATE I

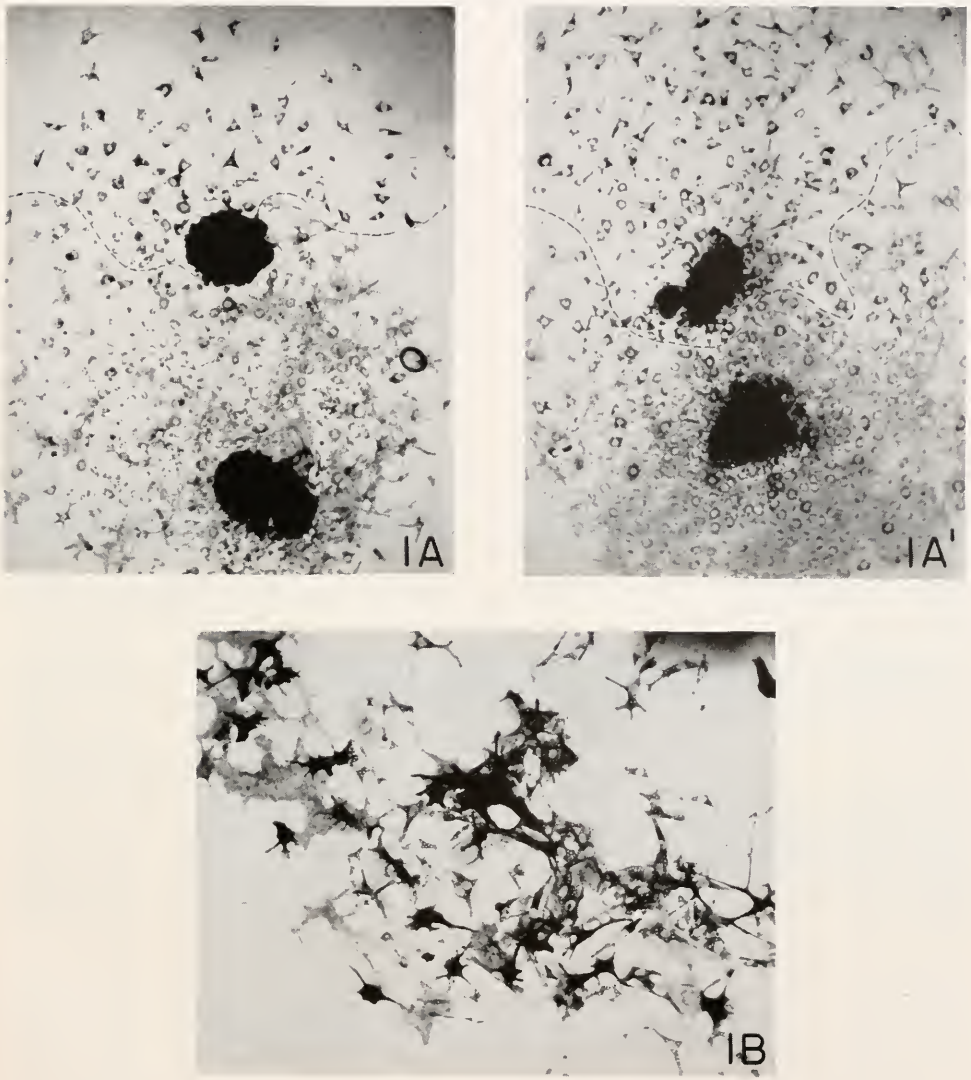


FIGURE 1A. A culture in Holtfreter solution of two pieces of neural crest differing moderately in age, showing a primary outgrowth (below) of *A. punctatum* chromatophores 11 days after explantation, and a secondary outgrowth (above) of *T. torosus* pro-pigment cells heavily charged with yolk platelets 5 days after explantation. A broken line marks the approximate boundary between the cells of the two outgrowths.

FIGURE 1A'. A different culture from the same series as figure 1A, 3 days later.

FIGURE 1B. A culture of two pieces of *T. torosus* neural crest of widely different age. Fully differentiated melanophores and light gray xanthophores 16 days after explantation can be seen with many pro-pigment cells of the secondary explant (6 days after isolation) migrating over them.

in inverted depression slides and three to four days were allowed for attachment of explants before the slides were righted and examined. The cultures were kept at 18° C. for the duration of the experiment.

The tendency for cells to migrate *in vitro* was taken as the indication of the presence of intercellular antagonisms. Using this basis for judgment, it appears that the initial development of pronounced intercellular antagonisms coincides with the onset of normal migration. The basis for this assumption is found in the commonly observed fact that a longer period of time was required for explanted neural folds to give outgrowths than was needed by neural crest. In either case, outgrowths in cultures did not begin until the explants reached an age approximating that at which pro-pigment cells normally began to leave the neural crest in control embryos. According to Detwiler (1937), this corresponds to the period between Stages 28 and 31 for *A. punctatum*, and it is assumed that approximately the same holds true in the other species used.

A simple modification of an experiment devised by Twitty (1945) was used in attempting to discover the duration of the period in which pigment cells actively repel one another. Instead of placing two explants of neural crest of the same age in a single drop of medium, pieces of neural fold were introduced into hanging drops of Holtfreter solution which possessed a previously explanted fragment of neural crest with its established outgrowth of cells (hereafter referred to as the "primary outgrowth"). By means of glass needles, the younger "secondary" explant was moved as near as possible to the edge of the primary outgrowth. The fluid in the drop was replenished before being re-sealed in an inverted depression slide. The cultures were not disturbed for three or four days to permit the secondary explants to become firmly attached to the glass surface. From 24 to 70 preparations of each of the following double explant combinations were made: *T. torosus* on *T. torosus*, *T. torosus* on *A. punctatum*, *A. punctatum* on *A. punctatum*, and *T. rivularis* on *A. punctatum*. In some cases the primary outgrowths were relatively young, and in others they were in advanced stages of differentiation when the secondary explant was added.

The results of Series 1 can be briefly summarized by saying that when the primary and secondary outgrowths differed only moderately in age (*i.e.*, 4 to 8 days), there was little mixing of cells when the margins of young and old outgrowths came in contact with one another. This is illustrated in Figures 1A and 1A′. The opaque areas in the figures show the positions of the explants (primary below, secondary above), and the broken line in each figure marks the approximate boundary between cells of the primary and secondary outgrowths. It will be noted that cells of the latter have migrated extensively only in previously unoccupied areas. This is taken as evidence for the existence of intercellular antagonisms which enable the partially differentiated cells of the primary outgrowth to halt the advance of younger cells. Fully mature chromatophores, on the other hand, offered virtually no impediment to the migration of young cells. This can be seen in Figure 1B in which young cells, abundantly supplied with yolk platelets, have migrated freely over the heavily pigmented cells of the primary outgrowth.

There is thus a clear indication that, *in vitro* at least, incompletely differentiated melanophores offered a more effective barrier to the migration of young pro-pigment cells than was presented by mature melanophores. More striking confirmation of this generalization was provided in the following series of transplantations.

Series 2. Duration of mutual antagonisms between chromatophores in vivo: In order to discover whether the age and distribution of older neural crest cells would influence the migration of younger cells within the embryo, three series of transplantations were carried out. They involve the grafting of identical pieces of Stage 23 *A. punctatum* trunk neural crest unilaterally onto the flank at the yolk border of Stage 24, 32, and 37 + *T. rivularis* embryos.

Series 2a. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 24 *T. rivularis* hosts: At the time of operation, both donor and host pro-pigment cells were at essentially the same developmental age. However, owing to the intrinsically slow pace of *T. rivularis* development, the host cells subsequently lagged considerably behind those of donor origin. Donor cells spread radially from the graft and achieved a wide distribution over the yolk mass. They appropriated areas anterior and posterior to the graft and extended as far dorsally as the base of the fin (Fig. 2A). Host cells were prevented from making an appearance in these areas until after Stage 41.

PLATE II

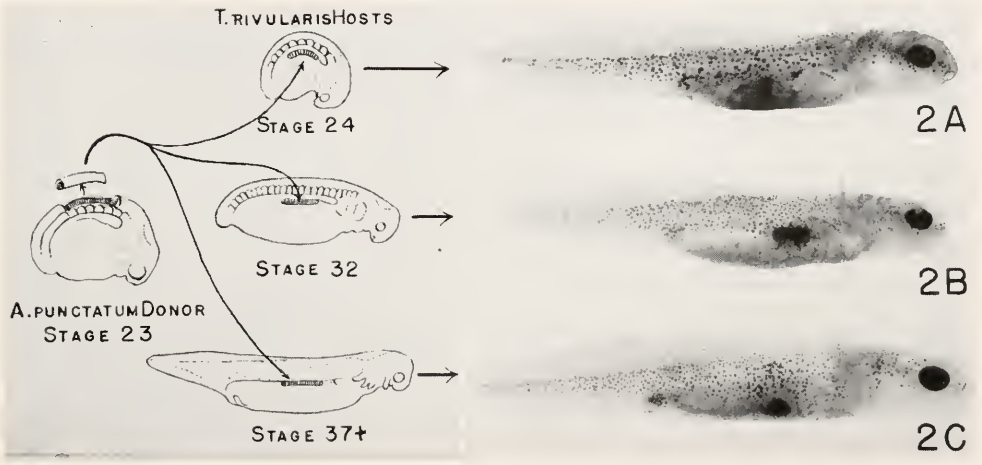


FIGURE 2A. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 24; figure 18 days after operation, at Stage 44 +.

FIGURE 2B. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 32; figure 8 days after operation, at Stage 42.

FIGURE 2C. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 37 +; figure 11 days after operation, at Stage 45. See explanation in text.

Series 2b. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 32 *T. rivularis* hosts: At the time of operation, host cells were in an early stage of active migration over the flanks, but as yet were not pigmented. By host Stage 36, a full complement of *T. rivularis* host cells was faintly visible on the flank dorsal to the graft before any donor melanophores appeared. When the latter did make an appearance, their distribution was restricted to the immediate vicinity of the transplant and to the surface of the yolk mass ventrolateral to the graft site (Fig. 2B). These results are noteworthy, inasmuch as they clearly demonstrate that, if given a moderate advantage in age, *T. rivularis* melanophores are able to inhibit the

migration of *A. punctatum* pro-pigment cells as effectively as they themselves were inhibited by *A. punctatum* cells in Series 2a.

Series 2c. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 37 + *T. rivularis* hosts: The pigmentation of host melanophores was well advanced at the time *A. punctatum* neural crest was grafted on the flank. Donor pigment cells were first observed at host Stage 40 − and, as can be seen in Figure 2C, they were able to migrate radially in all directions from the transplant. This result obtained in spite of the presence of fully mature host cells dorsal to the graft. Therefore, although the distance of migration was less extensive, the distribution of donor melanophores was similar to that achieved by *A. punctatum* cells in Series 2a.

The results from transplantations in Series 2 are in complete accord with those obtained from double explantations in Series 1. Series 2a and 2b duplicate *in vivo* the results obtained in Series 1 cultures in which the two explants differed only moderately in age and exhibited strong mutual antagonisms between cells of opposing outgrowths. Series 2c, on the other hand, is comparable to those explantations in which the fully differentiated cells of the primary outgrowth offered little opposition to invasion by young pro-pigment cells.

Series 3. Experimental modification of the degree of pigment suppression obtained in chimeric-crest combinations: Additional evidence concerning the relationship of intercellular antagonism to pigment suppression was obtained by modifying the standard procedure in preparing "chimeric-crest embryos" (*i.e.*, embryos in which one trunk neural fold was replaced at Stage 17 by a corresponding fold from another species; as a consequence, one side of the resultant neural crest is of donor and the other of host origin; see Lehman, 1950).

Series 3a. Development of *A. mexicanum* → *T. torosus* chimeric-crest embryos at low temperature: This chimeric-crest combination was selected because it had been shown (Lehman, 1950) that *A. mexicanum* chromatophores (which possess a developmental rate intermediate between those of *A. tigrinum* and *A. punctatum*) were most effective in suppressing the differentiation of Triturus melanophores. By subjecting *A. mexicanum* → *T. torosus* chimeric-crest embryos to low temperature following the operation, it was found that the high degree of inhibitory action of *A. mexicanum* chromatophores could be greatly minimized. Embryos kept for 30 days at 6° C. had advanced from Stage 17 to Stage 29–30 when they were finally returned to standard temperatures (14 to 18° C.). The degree to which *T. torosus* pigmentation was suppressed following this prolonged and severe chilling was markedly reduced. Figure 3A shows a normal *T. torosus* larva and Figure 3A′ shows a cold treated *A. mexicanum* → *T. torosus* chimeric-crest host. It will be noted that *T. torosus* melanophores on the experimental animal are only slightly fewer in number than those on the control. The contrast between these results and those obtained in chimeric-crest larvae reared at standard temperatures is evident by comparing Figures 3A′ and 3A″. In the latter, many more *A. mexicanum* pigment cells have differentiated and the suppression of *T. torosus* melanophores throughout the trunk region is essentially complete. This result obtained in spite of the presence of *T. torosus* neural crest along the entire anteroposterior axis of the host embryo.

This experiment demonstrates that the degree of suppression in chimeric-crest larvae is subject to modification by special treatment. However, it is not known

whether cold treatment resulted in an increase or a decrease in the difference between donor and host developmental rates. It is possible that the two components of the chimeric-crest were affected unequally or were retarded to such a degree that the normal disparity in rates of development was, in effect, eliminated or altered sufficiently to permit partial *T. torosus* pigmentation.

Series 3b. Chimeric-crest transplantations involving donor and host neurulae of different developmental age: The *A. punctatum* → *T. rivularis* combination was selected as offering the most favorable opportunity for observing the effects of slight alterations in the disparity between normal donor-host developmental rates. The difference between the developmental rates of these two species is relatively large, but has been shown (Lehman, 1950) to be slightly sub-optimal for obtaining a maximal degree of suppression. In order to reduce the difference between the developmental rates of donor and host tissues as much as possible, a trunk neural fold from young (Stage 14 −) *A. punctatum* neurulae was grafted in substitution for a neural fold on *T. rivularis* hosts in which the neural folds were just beginning to fuse (Stage 19 +). It was thought that by this means a sufficient age handicap might be imposed upon the grafted *A. punctatum* cells to reduce significantly their capacity to suppress *T. rivularis* pigmentation. The results are illustrated in Figure 3B′. It will be noted that *T. rivularis* melanophores are scattered abundantly over the flanks and fin. The more intensely pigmented *A. punctatum* melanophores are, in the main, aggregated ventrolaterally near the yolk border. Compare this distribution of donor and host cells with that in Figure 3B″, which is typical of *A. punctatum* → *T. rivularis* chimeric-crest larvae in which donor and host embryos were at the same developmental age (Stage 17) at the time of operation. The unusual arrangement of pigment cells shown in Figure 3B′ can be accounted for by assuming that *A. punctatum* cells were the first to migrate from the neural crest but were very shortly followed by host pro-pigment cells. The second wave, composed of *T. rivularis* cells, may be visualized as exerting mutual antagonisms which not only resulted in their own dispersion, but also kept the *A. punctatum* cells advancing peripherally until pigmentation took place and migration stopped.

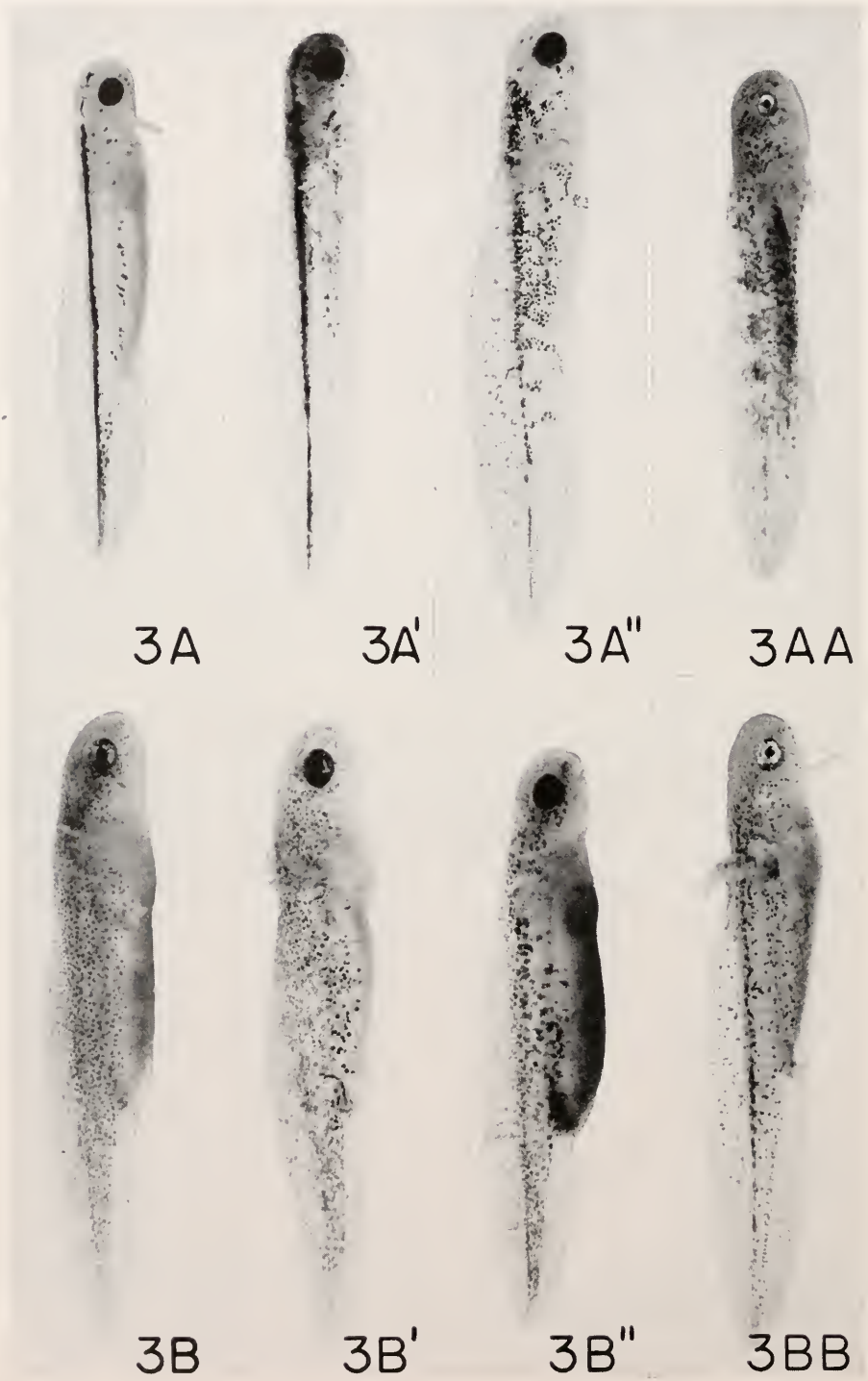
The experimental evidence provided by Series 1, 2, and 3 indicates that the period during which developing chromatophores exhibit antagonistic properties begins with the onset of migration, is most pronounced while the cells are becoming pigmented, and declines as melanophores approach complete histological differentiation. However, since they lose this character as they mature, why is it that in some Amblystoma—Triturus chimeric-crest combinations the suppression effect persisted long after the Amblystoma cells were fully pigmented?[3] The analysis of the factors responsible for this "secondary" delay in the differentiation of Triturus melanophores is the primary concern of the next section.

B. Influence of environmental factors on chromatophore differentiation

Many experiments have revealed that the ability of pro-pigment cells to synthesize melanin is in a large measure governed by the tissues surrounding them

[3] *T. torosus* melanophores, if failing to differentiate during the normal period before Stage 41, did not appear until the 18–20 mm. larval stage; *T. rivularis* melanophores, conversely, began to appear in Amblystoma pigment areas as early as Stages 41 to 44 and became progressively more abundant as development proceeded (Lehman, 1950).

PLATE III



(see reviews by DuShane, 1943 and 1948; Rawles, 1948; Twitty, 1949; and Lerner and Fitzpatrick, 1950). The precise nature of the environmental contribution to pigmentation is problematical; however, the most prevalent suggestion is that the epidermis and mesoderm supply hypothetical substances that act either as precursors of melanin or have some enzymatic action affecting the production of this pigment. An alternate theory has recently been advanced (Dalton, 1949 and 1950) to account for differences between white and black axolotls; it suggests that "the primary inhibitory action of white tissues was not on melanin synthesis but on the migration of pro-pigment cells" (1950, p. 152). Since the general applicability of Dalton's "migration-inhibition hypothesis" is still conjectural, the terminology of the more familiar "precursor-enzyme" hypothesis will be used in the following. The term "melanogenic substances" will be used to designate materials of unidentified nature and action which foster melanogenesis.

The problem at hand concerns the question raised at the close of the preceding section: namely, what is the basis for the secondary delay in Triturus melanophore differentiation after the pigmentation of Amblystoma cells was apparently complete on chimeric-crest embryos (for specific description, see Lehman, 1950)? If the "suppressed" Triturus cells were able to migrate onto the flanks after the fully pigmented Amblystoma cells had lost their antagonistic character, the secondary delay might be explained by: 1) a decline in the capacity of older epidermis to promote pigmentation, and/or 2) the depletion of melanogenic substances to subthreshold levels by Amblystoma cells which pre-empted available terrains at an earlier time. In either case, the question is reduced to whether or not the availability of melanogenic materials was associated with the secondary delay in pigmentation after intercellular antagonism could no longer be effective in suppressing the differentiation of these cells.

Series 4. Explantation of A. mexicanum → T. torosus chimeric-crest in peritoneal fluid: In an attempt to throw light on the above question, neural crest from *A. mexicanum → T. torosus* chimeric-crest embryos was cultured in peritoneal fluid obtained by abdominal puncture from spawning female salamanders. This culture medium was used, inasmuch as Twitty and Bodenstein (1939) had shown it to be more effective in promoting pigmentation *in vitro* than physiological salt solutions. It was reasoned that if melanogen concentration played any part in secondarily delaying pigmentation, it should be possible to induce some *T. torosus* cells to become pigmented by providing a rich melanogenic environment for their

PLATE III

FIGURE 3A. Normal pre-feeding *T. torosus* larva at Stage 40+.

FIGURE 3A′. Cold treated *A. mexicanum → T. torosus* chimeric-crest host at Stage 42 (explanation in text).

FIGURE 3A″. Stage 45 *A. mexicanum → T. torosus* chimeric-crest host reared at 14 to 18° C.

FIGURE 3AA. Normal pre-feeding *A. mexicanum* larva at Stage 41+.

FIGURE 3B. Normal pre-feeding *T. rivularis* larva at Stage 42.

FIGURE 3B′. Stage 14 − *A. punctatum* → Stage 19+ *T. rivularis* chimeric-crest host; figured at Stage 42 (explanation in text).

FIGURE 3B″. *A. punctatum → T. rivularis* chimeric-crest host in which both donor and host embryos were at Stage 17 at time of operation; figured at Stage 41.

FIGURE 3BB. Normal pre-feeding *A. punctatum* larva at Stage 44+.

development. The value of using this particular chimeric-crest combination is apparent from the fact that *in vivo* none, or at best very few, of the *T. torosus* propigment cells would have differentiated before the 18–20 mm. larval stage (Lehman, 1950).

The chimeric-crest embryos which provided neural crest for this experiment were prepared in the usual manner. After the orthotopically grafted *A. mexicanum* trunk neural fold had healed in place and neurulation was complete, the dorsal epidermis was stripped away. The exposed chimeric-crest was removed and divided into three pieces of equal size. Forty-three pieces were isolated in drops of

PLATE IV

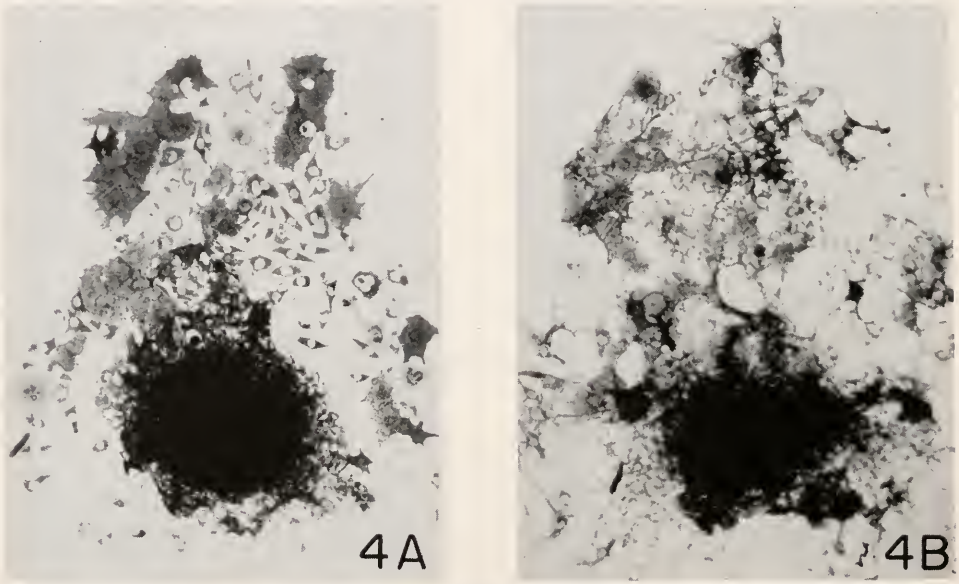


FIGURE 4A. A culture of *A. mexicanum* → *T. torosus* chimeric-crest in peritoneal fluid; figured 4 days after explantation. Well-differentiated *A. mexicanum* melanophores and xanthophores are peripherally located in the outgrowth. Small spindle shaped *T. torosus* propigment cells are easily recognized by their large yolk reserves.

FIGURE 4B. The same culture shown in figure 4A seven days after isolation. *T. torosus* cells are partially pigmented.

peritoneal fluid and kept at 18° C. for the duration of the experiment. After three days, outgrowths consisting of both *A. mexicanum* and *T. torosus* cells were well established. The *A. mexicanum* cells were for the most part located peripherally, thereby indicating that they had probably migrated from the explant in advance of the *T. torosus* cells. Figure 4A shows a four day culture in which *A. mexicanum* chromatophores are already well differentiated, whereas *T. torosus* cells are as yet unpigmented. In this photograph, small spindle shaped *T. torosus* cells appear dark, owing to the bi-refringence of numerous yolk platelets in the cytoplasm. *A. mexicanum* pigment cells are readily distinguishable from them by their larger size and smaller yolk reserves. Figure 4B shows the same culture three days later

after the *T. torosus* cells were partially pigmented. They later became fully differentiated but are not figured, since that by that time the majority of *A. mexicanum* cells had either become punctate or had detached themselves from the glass surface and presented a poor photographic subject.

One can conclude from Series 4 that the temporary association of Triturus and Amblystoma pro-pigment cells in chimeric-crest combinations does not significantly diminish the capacity of Triturus melanophores to migrate from the neural crest. Nor does it prevent their becoming pigmented *in vitro* when an excess of melanogenic material is available.

Series 5. Transplantation of Amblystoma epidermis on T. torosus chimeric-crest host: Amblystoma epidermis, which is more strongly melanogenic than that of *T. torosus* (DeLanney, 1941), was grafted on the flanks of chimeric-crest *T. torosus* hosts. This was done in an attempt to improve the regional conditions for pigmentation to such a degree that latent *T. torosus* melanophores, if present in the area, would become pigmented. It should be pointed out that early in their development *T. torosus* melanophores are uniformly distributed over the flanks and later re-aggregate along the dorsal border of the somites (Figs. 3A and 5A) as a response to the region of highest melanogenic activity in the embryo (Twitty, 1945). In this experiment, advantage was taken of the fact that the dorsal re-aggregation can be prevented if melanogenic conditions are sufficiently improved elsewhere as, for example, under grafted Amblystoma epidermis.

Series 5a. Orthotopic transplantation of Stage 27 *A. punctatum* flank epidermis on Stage 27 *A. punctatum* → *T. torosus* chimeric-crest embryos: The suppression phenomen was in general poorly expressed in this chimeric-crest combination and an appreciable number of host cells were regularly identifiable along the dorsal somite border (Fig. 5A′). It was therefore not surprising to find that host cells also appeared on the flanks at the site of epidermal grafts. One case from this series is shown in Figure 5A′, in which the location of the flank graft has been marked by a broken line. Most of the host cells within the area are in the contracted phase and appear as small dense points. This condition is not uncommon when differentiation takes place over the yolk mass as has occurred here. This experiment does not give any indication as to whether the *T. torosus* melanophores under the epidermal grafts represent, 1) latent melanophores which otherwise would have been suppressed, or 2) pro-pigment cells which merely failed to undergo secondary retraction to the dorsal somite border. Although no additional information is available, it is thought that the second alternative is probably correct.

Series 5b. Orthotopic transplantation of Stage 27 *A. mexicanum* flank epidermis on Stage 27 *A. mexicanum* → *T. torosus* chimeric-crest embryos: The strong melanogenic character of *A. mexicanum* epidermis was evidenced by a pronounced increment in the number of donor melanophores in the graft areas (Figs. 5B and 5B′). *T. torosus* pigment cells were not identified in the graft region except in those instances in which the transplanted epidermis extended dorsally beyond the base of the fin. In the latter case (Fig. 5B″), a few *T. torosus* melanophores frequently were observed under the grafted epidermis along the dorsal border of the somites. The ability of *A. mexicanum* epidermis to foster melanization in *T. torosus* cells *only* near the mid-dorsal line is interpreted to mean that *A. mexicanum* melanophores probably prevented the outgrowth of *T. torosus* pro-pigment cells

until early pre-feeding larval stages. If this were not the case, it would be difficult to account for the failure of *T. torosus* melanophores to respond to the strong melanogenic influence of the grafted epidermis as was the case in Series 5a.

It is probably safe to assume that availability of melanogenic substances was involved in the secondary delay in the differentiation of host cells in *A. mexicanum* → *T. torosus* chimeric-crest larvae, since some *T. torosus* melanophores did become pigmented *in vitro* (Series 4) and *in vivo* (Series 5b) when provided with a rich melanogenic environment.

Series 6. Transplantation of young and old ventral epidermis mid-dorsally on T. torosus tail-bud embryos: It was suspected that the temporal decline in the melanogenic strength of *T. torosus* epidermis described by Twitty (1936) might also contribute to the secondary delay in pigmentation of *T. torosus* cells in chimeric-crest embryos. With this in mind, Twitty's experiments were repeated for the purpose of determining the stage at which the melanogenic activity of the epidermis falls to subthreshold levels for *T. torosus* pigmentation.

The dorsal epidermis covering the neural tube and mesodermal somites was removed from the mid-trunk region of early *T. torosus* tail-bud embryos. Special care was taken to prevent injury to the underlying neural crest and mesoderm. The region of extirpation was then covered by mesoderm-free epidermis taken from the belly region of *T. torosus* donors of graded age. Belly epidermis was used in order to minimize the likelihood of transferring pigment cells adhering to donor epidermis. This region is not only farthest from the neural crest, but also Twitty and Bodenstein (1939) and DeLanney (1941) have shown that a barrier of unidentified character for a time prevents the ventral migration of pigment cells below the yolk border. The graft site was readily identifiable in larval stages, owing to the failure of grafted belly epidermis to respond to the dorsal fin inductor (Figs. 6A and 6B). Three classes of combinations were prepared; each consisted of 12 experimental animals, along with appropriate donor and host controls. The developmental age of donor controls was recorded when the hosts reached Stage 35, which coincides with the first appearance of host melanophores on the flank. This was done for the purpose of establishing an index of the age difference between donor and host epidermis during the active period of host melanophore differentiation.

Series 6a. Stage 13 + *T. torosus* belly epidermis grafted on Stage 28 *T. torosus* tail-bud hosts: Three days following the operation at host Stage 35, pigment cells began to appear in donor and host areas. At this time, donor controls had progressed to Stage 30 +. Thereafter, host pigment cells continued to develop in an entirely normal manner and formed dorsal bands which were essentially similar to those at non-graft levels and on host controls (Fig. 6A). The competence of young epidermis to foster pigmentation is apparently as great as that of the slightly older host epidermis.

Series 6b. Stage 33 *T. torosus* belly epidermis grafted on Stage 23 *T. Torosus* tail-bud hosts: In the second series, four days were required for the Stage 23 hosts to advance to Stage 35. During this interval, donor controls had progressed to Stage 38. Fewer melanophores per unit area became pigmented in the graft region than under host epidermis, but those which did appear were as darkly pig-

mented as those in non-graft areas. These results suggest that shortly after Stage 38, there is a decline in the melanogenic strength of *T. torosus* epidermis.

Series 6c. Stage 37 + *T. torosus* belly epidermis grafted on Stage 23 *T. torosus* tail-bud hosts: By the fourth post-operative day, pigment cells were visible on the Stage 35 host. By this time the donor controls had advanced to Stage 40 +. The number of melanophores that differentiated in graft areas was very drastically reduced and there were several instances in which their elimination was almost complete. This condition is shown in Figure 6B, in which the graft region has been outlined by a broken line. It should be mentioned that technical difficulties were encountered in transplanting Stage 37 + epidermis which has a strong tendency to curl. Healing was also slow and some of the host neural crest was lost before the wound was completely closed. It is possible that this may have caused a moderate reduction in the number of pigment cells in the graft region, but it is improbable that this factor could alone be responsible for the results obtained. Evidence for the presence of latent melanophores at graft levels was provided, moreover, by the presence of normal numbers of fully differentiated pigment cells around the neural tube, even though they were lacking under the overlying grafted skin. Melanophores around the neural tube are faintly visible through the myotomes in Figure 6B.

One can tentatively conclude that between Stages 38 and 40 + or shortly thereafter, *T. torosus* epidermis to a large measure loses its capacity to promote pigmentation in prospective *T. torosus* melanophores.[4] Thus, if rapidly developing Amblystoma pigment cells were able to delay the differentiation of *T. torosus* cells until Stage 41 (that is, by intercellular antagonisms that prevented outgrowth or by reducing melanogens to sub-threshold levels), then ageing of the *T. torosus* epidermis itself could become a significant factor contributing to a secondary delay in the pigmentation of *T. torosus* melanophores.

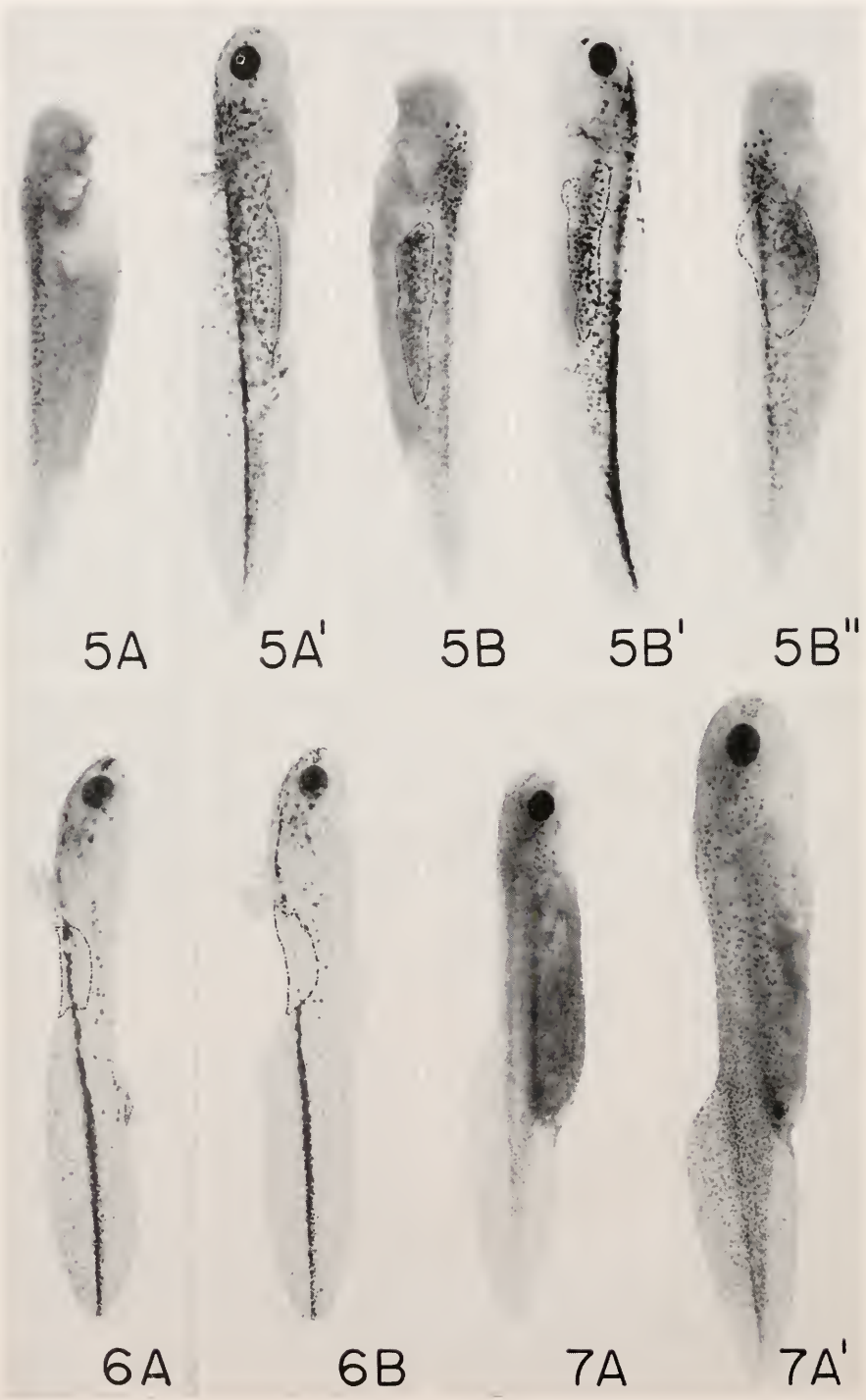
C. Intrinsic species differences in pro-pigment cell behavior

The question to receive immediate attention concerns the possibility that irrespective of the melanogenic character of surrounding tissues, the period and sequence of melanophore differentiation may be independently controlled by factors intrinsic to the pigment cells themselves. This possibility was suggested by differences in the normal manner of pigmentation in *T. torosus* and *T. rivularis* embryos, and by differences in the duration of the secondary delay in Triturus pigmentation on chimeric-chest embryos.

Series 7. Sequence of melanophore differentiation in species of Triturus and Amblystoma: The manner in which regions deprived of trunk neural crest became progressively invaded by melanophores from outlying regions provided the basis for distinguishing species differences in the time and sequence of melanophore differentiation. Trunk crest-less embryos were prepared by removing both trunk neural folds from Stage 16–17 neurulae. Following the operation, closure of the neural tube was essentially normal. Subsequent development was also normal ex-

[4] For the present, this interpretation has ignored a possible source of error which is currently being investigated, namely, the question of whether or not the time and degree of loss in melanogenic strength are similar in dorsal and ventral epidermis.

PLATE V



cept for the absence of neural crest derivatives and a dorsal fin in the region of extirpation. In all species used, the crest-less area remained free of differentiated pigment cells until Stage 39. However, the region eventually became populated by melanophores, and the sequence in which this was accomplished was taken as a measure of the inherent capacity of pro-pigment cells in each species to continue to differentiate over an extended developmental period.

Series 7a, 7b, and 7c dealt respectively with the development of trunk crest-less embryos of *T. rivularis* (Figs. 7A and 7A′), *A. mexicanum* (Figs. 7B and 7B′), and *A. punctatum* (Figs. 7C and 7C′). In these species, the invasion of crest-less areas by melanophores originating from levels anterior and posterior to the region of extirpation began at about Stage 40 and continued until the region was uniformly populated by pigment cells. Depending upon the anteroposterior extent of the crest-less area, pigmentation was completed at any time from pre-feeding to mid-larval stages of development. It was concluded that melanophore differentiation in these species can continue almost uninterruptedly over a major part of the developmental period.

Series 7d was concerned with trunk crest-less *T. torosus* embryos (Figs. 7D and 7D′), and provided results in sharp contrast to those obtained in the preceding series. The sequence of pigmentation in trunk crest-less animals closely paralleled that observed in normal *T. torosus* larvae in which two distinct populations of melanophores are recognized (*viz.*, the "primary melanophores" which appear between Stages 34 and 41 and re-aggregate into well defined dorsal bands that constitute the primary pigmentation of pre-feeding larvae, and the "secondary melanophores" which are smaller, less heavily pigmented cells that appear after the 18 to 20 mm. larval stage and permanently retain a random distribution on the fin and flanks. See Figures 8B and 8C, and Lehman, 1950). "Primary" *T. torosus* melanophores did not invade crest-less areas to any appreciable extent and retained the distribution achieved by Stage 39 (Fig. 7D). No additional pigmentation of crest-less regions occurred until the larvae reached a length of approximately 18

PLATE V

FIGURE 5A. Normal *T. torosus* embryo at Stage 36+, showing melanophores in the process of retracting toward the dorsal border of the somites.

FIGURE 5A′. *A. punctatum* → *T. torosus* chimeric-crest host bearing a flank graft of *A. punctatum* epidermis; site of graft indicated by broken line; figured at Stage 43.

FIGURE 5B. *A. mexicanum* → *T. torosus* chimeric-crest host bearing a flank graft of *A. mexicanum* epidermis; figured at Stage 37.

FIGURE 5B′. Same embryo shown in figure 5B, at Stage 41. Explanation in text.

FIGURE 5B″. Stage 37 embryo from the same series as figure 5B. The presence of darker host melanophores along the dorsal border of the myotomes, under grafted *A. mexicanum* epidermis that extended beyond the base of the fin, is shown.

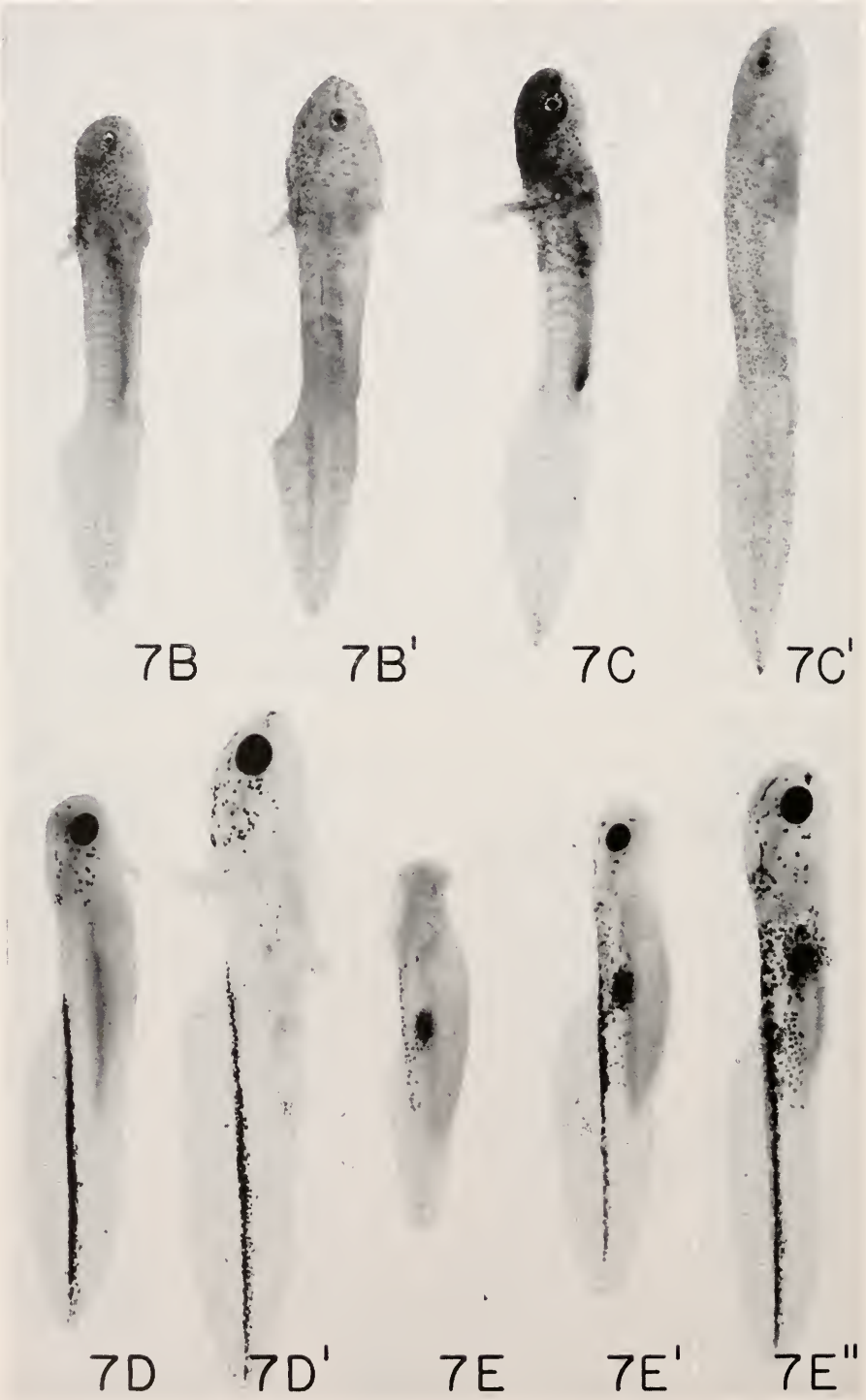
FIGURE 6A. An 18 mm. *T. torosus* larva in which Stage 13 + *T. torosus* belly epidermis was grafted mid-dorsally at Stage 28. Site of graft indicated by broken line.

FIGURE 6B. An 18 mm. *T. torosus* larva in which Stage 37 + *T. torosus* belly epidermis was grafted mid-dorsally at Stage 23. Explanation in text.

FIGURE 7A. Trunk crest-less *T. rivularis* embryo at Stage 40. Note deficiency in the dorsal fin and the absence of melanophores in the region of extirpation.

FIGURE 7A′. Embryo similar to that in figure 7A at Stage 45. The crest-less area has become populated by melanophores that have migrated into it from outlying region (original photograph by V. C. Twitty).

PLATE VI



mm. (Fig. 7D′). Thereafter, melanophore differentiation continued slowly through the remainder of the larval period. So far as could be determined on the bases of appearance and distribution, the gradual invasion of crest-less areas was accomplished entirely by "secondary" melanophores.

Series 7e demonstrated that *A. tigrinum* embryos, like those of *T. torosus,* have distinct "primary" and "secondary" generations of melanophores, which are recognizable on the bases of differences in time of pigmentation and definitive distribution. *A. tigrinum* embryos in neurula stages were not available and, instead, tail-bud embryos were used in the following manner. A graft of Stage 25 *A. tigrinum* neural crest was placed on the flank of Stage 32 *T. torosus* hosts which previously had been deprived of both trunk neural folds, so as to free the trunk region of host chromatophores. By the fourth post-operative day, donor melanophores were well differentiated on the trunk crest-less *T. torosus* hosts, which by this time had advanced to Stage 37. As can be seen in Figure 7E, the "primary" donor melanophores were aggregated along the mid-dorsal line or near the flank graft. Host cells are faintly visible in the tail. On the tenth post-operative day, at host Stage 40, "secondary" donor melanophores began to appear (Fig. 7E′). They were distinguishable from those that appeared earlier by permanently retaining a widespread distribution on the flanks. Their numbers gradually increased until both sides of the *T. torosus* hosts were densely populated (Fig. 7E″).

The above observations have the advantage of clearly showing that the discontinuous sequence of melanophore differentiation in *A. tigrinum* results from inherent differences in the developmental rates of primary and secondary melanophores, and is not due to the intervention of known environmental factors, as might be the case in *T. torosus* larvae. This conclusion is tenable, since the appearance of secondary *A. tigrinum* melanophores on Stage 40 + *T. torosus* hosts coincided in time with a known decline in the melanogenic strength of the host epidermis (see Series 6). So far, then, as the tissue environment of the secondary *A. tigrinum* pro-pigment cells was concerned, conditions were more favorable for pigmentation during host Stages 37 to 40, in which melanization failed to take place. This can be correlated with the fact that they are less "dependent" (DeLanney, 1941) upon environmental factors for pigmentation than are the cells of *T. torosus.* The interrupted sequence of *T. torosus* pigmentation also indicates that there are inherent differences between the primary and secondary melanophores in this species. However, it remains in question whether the differences concern

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PLATE VI

FIGURES 7B AND 7B′. Stage 41 and 16 mm. larval stage of a trunk crest-less *A. mexicanum* larva; pigmentation essentially as in figures 7A and 7A′.

FIGURES 7C AND 7C′. Stage 45 and 23 mm. larval stage of a trunk crest-less *A. punctatum* larva; pigmentation essentially as in figures 7A and 7A′.

FIGURES 7D AND 7D′. Stage 40 and 18 mm. larval stage of a trunk crest-less *T. torosus* larva. The region of extirpation remained essentially free of melanophores until shortly after this period. A few secondary melanophores are visible at the base of the fore limb in figure 7D′.

FIGURE 7E. A trunk crest-less *T. torosus* host bearing an *A. tigrinum* neural crest graft on the flank; figured 7 days after operation. Explanation in text.

FIGURE 7E′. The same embryo shown in figure 7E, 13 days after the operation.

FIGURE 7E″. The same embryo shown in figures 7E and 7E′, 21 days after the operation.

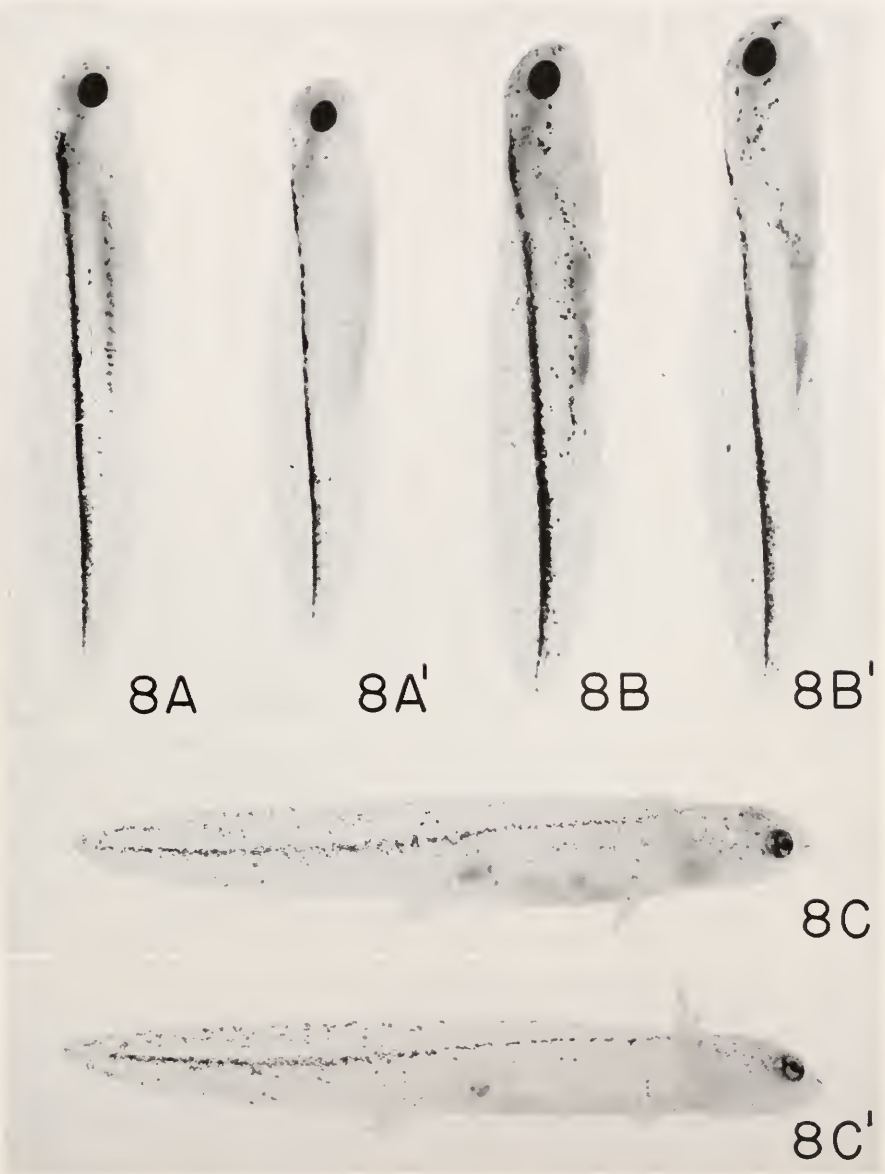
rates of development or differences in sensitivity to environmental factors such as melanogenic materials or larval hormones. Unfortunately, the evidence on hand does not provide an answer to this question. Possibly both an intrinsic limitation of the period of differentiation, and the declining melanogenic strength of the epidermis contribute to the failure of primary *T. torosus* melanophores to develop except during Stages 32 and 41. If this were indeed the case, the temporal coincidence in the action of intrinsic and environmental factors would provide an example of the well known principle of "double assurance" (Braus, 1906, and Spemann, 1938, pp. 92–97) ; that is, either factor by itself might be able to prevent the differentiation of primary *T. torosus* melanophores after Stage 41.

Series 8. Unilateral extirpation of trunk neural folds from T. rivularis and T. torosus Stage 17 neurulae: The manner in which crest-less areas became pigmented in embryos of *T. rivularis* (Series 7a) and *T. torosus* (Series 7d) suggested the presence of yet another difference between these species. If one considers only those pigment cells which appear during pre-feeding stages, the impression is given that the capacity of neural crest to supply melanophores in excess of those normally differentiating is much greater in *T. rivularis* than in *T. torosus*. This possibility was examined by subjecting Stage 17 neurulae of both species to unilateral extirpation of one entire trunk neural fold. The operation did not interfere with the closure of the neural tube and by this means it was possible to reduce the amount of tissue comprising the neural crest to one-half that found in normal embryos. Embryos treated in this manner will hereafter be referred to as "half-crest"embryos or larvae.

When the pre-feeding larvae were later examined for deficiencies in the melanophore population, it was found that half-crest *T. rivularis* larvae were indistinguishable from normal animals with regard to number and distribution of pigment cells. Therefore, since one *T. rivularis* neural fold can give rise to a full complement of pigment cells, it is concluded that the regulatory capacity of *T. rivularis* neural crest is at least two times greater than the normal expression of its ability to produce pigment cells.

The number of *T. torosus* melanophores on half-crest embryos was conspicuously lower than that on normal larvae from the same egg clutch. In Figures 8A and 8A′, one will note that the dorsal band on the half-crest embryo is narrower than the band on the control. This is a direct reflection of the smaller number of primary melanophores present. The experimental animals moreover differed from the controls by lacking melanophores at the yolk border. The absence of melanophores at the yolk border can be explained by the fact that the distance travelled by a given pigment cell is roughly proportional to the number and proximity of other pigment cells (Twitty and Niu, 1948). Hence, with fewer propigment cells to exhibit mutual antagonisms, migration probably failed to proceed as far ventrally as the yolk border and all the cells were later withdrawn from the flanks in the formation of the dorsal bands. Cell counts made on 16 to 17 mm. larvae while the melanophores were in the contracted phase revealed that the number of primary melanophores in the dorsal bands of half-crest embryos was 30 to 40 per cent lower than the number found in normal larvae (Figs. 8B and 8B′). Thus, even though the single neural fold contributed more primary melanophores than it would have in a control embryo, the regulatory capacity of *T. torosus* neural

PLATE VII



FIGURES 8A, 8B, AND 8C. A normal *T. torosus* at pre-feeding larval Stage 41, and at 17 and 30 mm. larval stages respectively, showing the gradual appearance of secondary melanophores.

FIGURES 8A′, 8B′, AND 8C′. A half-crest *T. torosus* larva at corresponding stages, showing a reduction in the number of primary melanophores in the dorsal band.

crest presumably does not exceed 1.2 to 1.4 times the number of primary pro-pigment cells which normally differentiate. A limitation of this sort apparently does not apply to the quantity of secondary melanophores produced. The differentiation of these cells in half-crest larvae lagged only slightly behind that observed in controls (Figs. 8C and 8C′).

Discussion

The experimental analysis of amphibian pigmentation has at present progressed to a point that permits an array of interacting factors to be recognized as mediators in the final expression of chromatophore development (see review by Twitty, 1949). By acknowledging the complexity of the overall problem of pigmentation, it was possible to avoid the initial error of expecting that a single factor such as growth rate, age, or species differences might alone be responsible for the phenomenon of pigment suppression with which this paper is concerned. The following factors have been identified as collective determinants in pro-pigment cell differentiation: 1) the ability of partially differentiated melanophores to be more effective than mature cells in repelling invasion by younger pro-pigment cells, 2) genetic, regional, and temporal differences in the capacity of embryonic ectoderm and mesoderm to promote melanin synthesis, 3) a possible competition between chromatophores for terrain and melanogenic substances, and 4) species differences in the behavior of chromatophores with respect to, a) their dependence upon the environment for melanogenic substances, b) the sequence of melanophore differentiation during development, and c) the regulatory capacity of the neural crest to produce more than the normal number of pigment cells. It is apparent that as these conditions are altered by transplantation between embryos of different age or genetic constitution, there will be modifications in the degree to which the prospective potency of pro-pigment cells is realized. The degrees of pigment suppression obtained thus may vary from complete (in which the concerted action of extrinsic factors works to prevent the differentiation of one group of pro-pigment cells) to very indifferent manifestations of this phenomenon. It is necessary to realize that temporal changes continually alter not only the cellular environment but also the intrinsic capacities of the cells. Synchronized timing is therefore of cardinal importance in pigment suppression, since the results in a given instance are colored by the sequence with which each of the above factors comes into play, and independently changes as development proceeds.

Before proceeding to the interpretation of suppression in specific chimeric-crest combinations, it should be pointed out that the major contrast between the melanophores of *T. rivularis* and *T. torosus* is provided by a comparison of the cells of *T. rivularis* with the *"primary"* melanophores of *T. torosus*. If, instead, only the *"secondary" T. torosus* cells were considered, one would find that the similarities far outweigh the differences. For example, both *T. rivularis* and secondary *T. torosus* melanophores are characterized, 1) by being relatively independent of the environment for melanogenic substances, 2) by permanently retaining a random, widespread distribution, 3) by being highly regulatory with regard to numbers, and 4) by exhibiting continuous differentiation of new cells over much of the developmental period. These traits probably should be considered primitive chromatophore characteristics. Primary *T. torosus* melanophores, with their sen-

sitivity to melanogen fluctuations, their tendency to undergo re-aggregation, and their limitations in number and time of differentiation, suggest that here one is dealing with a highly specialized type of pigment cell (see Twitty, 1945, pp. 173–174).

The remainder of the discussion will attempt to define the probable roles of intrinsic and environmental factors influencing melanophore differentiation in the Amblystoma—Triturus chimeric-crest experiments (Lehman, 1950), which provided the point of departure for the present study.

Analysis of suppression in A. punctatum → T. rivularis chimeric-crest embryos: The suppression of *T. rivularis* melanophores was not striking in this combination and may be viewed simply as delayed pigmentation in which migration and melanogenesis were temporarily held in abeyance by the antagonistic action (see Series 1, 2, and 3) of rapidly developing *A. punctatum* cells. *T. rivularis* melanophores began to appear in flank areas almost as soon as *A. punctatum* melanophores had completed their differentiation at Stage 40 +. Until shortly before this time, they presumably were immobilized in the vicinity of the mid-dorsal line. The disparity between the developmental rates of the two species was apparently too slight to provide more than a transitory impediment to the outgrowth and differentiation of *T. rivularis* cells which have no sharply restricted period during which pigmentation must occur (Series 7a). The differentiation of *T. rivularis* pigment cells at a time when the epidermis had probably become weakly melanogenic (Series 6) can be correlated with the fact (DeLanney, 1941) that their differentiation is relatively "independent" of environmental factors, whereas *T. torosus* melanophores are very sensitive to variations in the melanogenic character of the surrounding tissues. Thus, a decline in the ability of ageing epidermis to foster pigmentation need not interfere appreciably with *T. rivularis* pigmentation, but still might be very effective in preventing the differentiation of *T. torosus* melanophores during pre-feeding larval stages.

Analysis of suppression in A. mexicanum → T. rivularis chimeric-crest embryos: The primary difference between this and the preceding series centered around the failure of *T. rivularis* melanophores to appear on the flank until approximately Stage 44 +. It is surmised that after Stage 39 (when donor *A. mexicanum* melanophores appeared to be fully differentiated and host cells were appearing in the dorsal fin), latent melanophores of both species were present on the flanks and thereafter competed, perhaps on equal footing, for the limited remaining terrain. Some unidentified intrinsic property, such as a more rapid division rate or stronger intercellular antagonisms, may have enabled *A. mexicanum* melanophores to "saturate" the terrain more completely than those of *A. punctatum* in the preceding series. As a result, there may have been less available space and sharper competition for melanogenic materials, which could account for the slight secondary delay in the appearance of *T. rivularis* melanophores.

Analysis of suppression in A. punctatum → T. torosus chimeric-crest embryos: The lowest level of pigment suppression was encountered in this combination. This is correlated with the fact that during the critical period between Stages 32 and 38, the developmental rates of the two species are very nearly equal (see Leh-

man, 1950, Figure 3). Therefore, it might be expected that the pro-pigment cells of both neural crest components would initiate migration and undergo pigmentation almost simultaneously. Under such conditions, intercellular antagonism between developing chromatophores would be responsible for the general spreading and dispersion of *T. torosus* and *A. punctatum* pro-pigment cells alike. The number of *T. torosus* melanophores which differentiated was approximately one-third the number appearing on normal embryos. This is not surprising when one considers that the removal of one *T. torosus* neural fold would, of itself, result in a 30 to 40 per cent reduction in the number of primary *T. torosus* melanophores developing (Series 8), irrespective of whether or not a fold from another species were added. Consequently, the suppression of primary melanophores in all *T. torosus* chimeric-crest larvae would appear to be more pronounced than is actually warranted. In *T. rivularis* combinations, however, the effect of eliminating one neural fold can, for all practical purposes, be discounted, since a single neural fold is capable of supplying a normal complement of pigment cells. The final factor to consider is the melanogenic strength of surrounding tissues which restricts the density of the melanophore population. Faced with an environmental limitation of this sort, it follows that donor and host cells of nearly equal developmental rates will act reciprocally upon each other in competing for space and melanogenic substances, thereby establishing a balance that holds in check the capacity of both donor and host cells to undergo melanization.

Analysis of suppression in A. mexicanum → T. torosus chimeric-crest embryos: Maximal inhibition of primary *T. torosus* melanophores was obtained in this combination and probably resulted from the concerted action of several factors. It was suggested "that the most favorable conditions for pigment inhibition are realized (as in *A. mexicanum → T. torosus* combinations) when the difference in rate of pigmentation is of such magnitude as to permit rapidly developing donor chromatophores to appear initially at approximately host Stage 31–32" (Lehman, 1950, p. 448). It now appears that this relationship obtains because, at the time at which *T. torosus* pro-pigment cells would normally begin to migrate from the neural crest at Stages 28 to 31, *A. mexicanum* pigment cells had already appropriated all available areas. More important, the *A. mexicanum* cells were at the stage during which the capacity to prevent invasion was at a peak (Series 1, 2, and 3). Consequently, during the initial migratory period *T. torosus* melanophores were probably unable to gain access to the flank terrain (Series 5b). *A. mexicanum* melanophores were visible on the flanks by Stage 32 and new cells were evidently able to differentiate continuously during much of the larval period (Series 7b). After Stage 41, the intrinsic inability of primary *T. torosus* melanophores to become pigmented (Series 7d), or the temporal decline in the melanogenic strength of the epidermis (Series 6), would be sufficient to prevent the appearance of primary host cells. Competition between donor and host cells was probably not an important factor in this combination. The only region in which it probably occurred was along both sides of the mid-dorsal line where some intermingling of donor and host cells very likely took place prior to Stage 41. The actual fate of latent primary *T. torosus* cells is as yet undetermined. However, it is likely that after failing to achieve their prospective fate, they enter into some other channel of neural crest development, *e.g.*, secondary melanophores, neurons, mesenchyme, etc.

Analysis of suppression in A. tigrinum → T. torosus chimeric-crest embryos: The degree to which *T. torosus* primary melanophores were suppressed by *A. tigrinum* cells was less than that encountered in the preceding series, even though *A. tigrinum* embryos developed appreciably more rapidly than those of *A. mexicanum*. In order to explain this paradox, it is necessary to make the assumption that *A. tigrinum* melanophores became fully differentiated before host Stage 41. That is to say, the donor cells must have lost the ability to repel host cells and were incapable of lowering chromogen concentration to sub-threshold levels while the primary *T. torosus* pro-pigment cells were still able to differentiate and while the epidermis was able to promote their pigmentation. It has been shown that the melanophores of *A. tigrinum* have an interrupted sequence of pigmentation (Series 7e), and that after the primary *A. tigrinum* melanophores became well pigmented, a period of five to six days elapsed before additional cells began to appear. Since the primary donor melanophores on *A. tigrinum → T. torosus* chimeric-crest embryos were fully differentiated by host Stage 35 (Lehman, 1950), the secondary donor cells probably began to appear at approximately host Stage 38. The few *T. torosus* melanophores which made an appearance probably did so during the interval between the two successive periods of donor differentiation. An argument in support of this view is provided by the observation that *T. torosus* melanophores were not seen until Stage 38. This is taken as evidence that the migration and pigmentation of these cells had temporarily been delayed until primary donor melanophores were in an advanced stage of development. However, even after Stage 38, the number of *T. torosus* melanophores which became pigmented was small. This perhaps should be expected when one recalls that a relatively short period remained after Stage 38, during which the epidermal strength was sufficient to permit pigmentation and the primary *T. torosus* cells were still able to respond. The final factor is the role played by secondary *A. tigrinum* melanophores. They very likely began to emerge simultaneously with the primary host cells at Stage 38, and active competition between them would tend to reduce still further the number of host cells which otherwise might have appeared.

It is appropriate, therefore, to point out that in the case of *A. tigrinum* and *T. torosus* embryos in which there are distinct primary and secondary melanophore generations, a condition exists in the normal embryo which is somewhat comparable to that in chimeric-crest embryos. Although experimental evidence is lacking, it is possible that in *A. tigrinum*, immature primary melanophores may prolong a normal slight delay in the migration and pigmentation of secondary cells. The prospective secondary melanophores of *T. torosus* may also be able to migrate onto the flanks *only* after the primary cells have completed their differentiation; however, owing to a melanogen deficiency, they remain unpigmented until some other factor (probably hormonal) comes into play and permits their gradual differentiation during larval stages. In the embryos of *A. mexicanum*, *A. punctatum* and *T. rivularis*, the sequence of chromatophore differentiation is apparently continuous, and no visible distinction can be made between early and later generations of pigment cells. Nevertheless, even in these species the initial population of melanophores may impose a partial barrier that retards the outgrowth and pigmentation of cells following after them. Owing to its regulatory capacity, the neural crest of most species is capable of providing many more chromatophores than normally become pigmented. The limiting factor in their development is established by the epidermis and mesoderm, which

govern the number of melanophores than can appear in a given area. One might consider that the pigment cells which do differentiate in normal development are those which have successfully competed for the limited facilities of the environmental terrain and thereby have, in effect, "suppressed" other cells in the same area. The prospective melanophores eliminated in the competition for space and melanogenic materials may eventually become pigmented, or be consigned to other channels of neural crest development. In either event, they very likely share the fate, whatever it may be, of cells similarly "suppressed" in chimeric-crest embryos.

Viewed in this light, pigment suppression in chimeric-crest embryos may be merely an accentuated expression of processes operating in normal development. That is, the grafted Amblystoma cells may simply appropriate the role normally played by the initial complement of Triturus melanophores, with the result that an increased percentage of host cells is forced to accept the status and fate of unpigmented prospective melanophores.

Summary

The present study was undertaken for the purpose of clarifying the developmental mechanics operating in the phenomenon of pigment suppression in salamanders. The experiments include the transplantation of epidermis and the explantation, transplantation and extirpation of neural folds and neural crest in five species of salamanders. These species, listed in the order of increasing rates of development, are: *Triturus rivularis*, *T. torosus*, *Amblystoma punctatum*, *A. mexicanum* and *A. tigrinum*.

1. By culturing two fragments of neural crest of different age in single drops of culture medium, it was demonstrated that incompletely differentiated melanophores offer a more effective barrier to the migration of young pro-pigment cells than do the cells of fully mature outgrowths.

2. The same relationship between intermediate stage of differentiation and maximal ability to prevent invasion by younger cells was demonstrated *in vivo* by transplanting young *A. punctatum* neural crest on the flanks of *T. rivularis* embryos of graded age.

3. It was found that the degree of pigment suppression obtained in "standard" chimeric-crest embryos (unilateral orthotopic grafts of trunk neural folds) could be reduced either by prolonged chilling of the embryos following the operation, or by using donor and host neurulae of different developmental age. It is assumed that in both instances, the normal difference in donor and host developmental rates was altered in such a manner that an optimal intermediate disparity was not realized.

4. Chimeric-crest taken from *A. mexicanum* → *T. torosus* embryos (a combination in which suppression is maximally expressed) and explanted in peritoneal fluid gave mixed outgrowths of *A. mexicanum* and *T. torosus* cells, both types of which eventually became pigmented. This result is noteworthy, inasmuch as had they developed *in vivo*, the *T. torosus* cells undoubtedly would have been suppressed.

5. Amblystoma epidermis which is "strongly melanogenic" was grafted on the flanks of chimeric-crest hosts as a means of testing for the presence of unpigmented *T. torosus* cells on the flanks. *T. torosus* melanophores did differentiate under epidermal flank grafts on *A. punctatum* → *T. torosus* hosts (a combination exhibiting a

low degree of suppression), but they failed to appear in *A. mexicanum* → *T. torosus* hosts except under special circumstances. It is concluded that *A. mexicanum* cells had prevented the outgrowth and differentiation of *T. torosus* pro-pigment cells.

6. Homoplastic transplantations of belly epidermis from *T. torosus* donors of graded age mid-dorsally onto tail-bud hosts revealed that between Stages 38 and 40 +, *T. torosus* epidermis loses most of its capacity to promote pigmentation in Triturus melanophores. It is suggested that this may be responsible for secondarily delaying the differentiation of *T. torosus* pro-pigment cells after the donor cells are well differentiated on chimeric-crest hosts.

7. Observations on the manner in which trunk regions deprived of neural crest became invaded by melanophores indicate that melanophore differentiation is essentially a continuous process in *T. rivularis*, *A. mexicanum* and *A. punctatum* embryos. In *T. torosus* and *A. tigrinum* embryos, the sequence of pigmentation was found to be interrupted, and consisted of distinct "primary" and "secondary" generations of melanophores.

8. It was noted that the removal of one trunk neural fold from *T. rivularis* embryos had no observable effect upon the number of pigment cells that later appeared. In similarly treated *T. torosus* embryos, however, this resulted in a 30 to 40 per cent reduction in the number of cells normally appearing.

The results of previous chimeric-crest experiments (Lehman, 1950) are analyzed in the light of the above findings.

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ENDOGENOUS OXYGEN UPTAKE AND SPECIFICITY OF EMBRYONIC INTRACELLULAR CONSTITUENTS [1]

JOSEPH HALL BODINE AND KIAO-HUNG LU

Zoological Laboratory, State University of Iowa, Iowa City

The action and interaction of the various internal parts of the living cell, especially as they relate to its normal functioning, have long been of interest to the experimental biologist. That the reactions of living cells are the results of enzyme-substrate relationships seems well established but many of the details of these phenomena are as yet not clear. The extent to which the nucleus controls or regulates the type or nature of chemical reactions occurring in the cell as a whole has to a great degree been discussed from a theoretical rather than from a scientific point of view (DeRobertis, Nowinski and Saez, 1950). The presence, distribution and localization of intracellular enzyme-substrates and chemical compounds have been variously demonstrated by micro-chemical and other techniques (Caspersson, 1950). The fact that living cells are in themselves able to carry on the reactions essential for their metabolic activities also seems well established. The degree to which the different parts of the cell are specific for these physico-chemical phenomena is at present not too well understood (Caspersson, 1950; Brachet, 1950). Details as to the presence of typical chemical compounds in the different parts of living cells are rapidly accumulating and tend to show a marked regional localization for many of the materials deemed essential for the various functions of the parts of the cell (Lardy, 1949; Caspersson, 1950). Results from many investigations on the enzyme-substrate relations of cellular parts seem to deal largely with systems isolated from the cells and to which various reagents have been added and, conclusions then deduced from results of such extracellular experiments, are referred to the possible conditions in the intact normal cell (Schneider, 1946; Schneider, Claude and Hogeboom, 1948; Lardy, 1949). Valuable data, however, have accumulated from investigations carried out by these methods. It seems reasonable to assume that investigations of the endogenous metabolism of the intact living cell and its parts should contribute additional data to an understanding of intracellular phenomena. To this end, extensive experiments have been carried out on the endogenous oxygen uptake of the various parts of the normal living embryonic cells of the grasshopper embryo. The embryo of the grasshopper (*Melanoplus differentialis*) has many advantages for such investigations since it can be rigidly controlled and standardized (Bodine and Lu, 1950a). Yolk and other extraneous materials

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which normally interfere to a marked degree in such experimental work can be practically eliminated. Since the embryo is a cold-blooded animal, rigid temperature and developmental controls can easily be maintained (Slifer, 1931). The present data are concerned with the endogenous oxygen uptake of the homogenates and intracellular parts of the embryonic cells of mitotically active (postdiapause) and developing embryos.

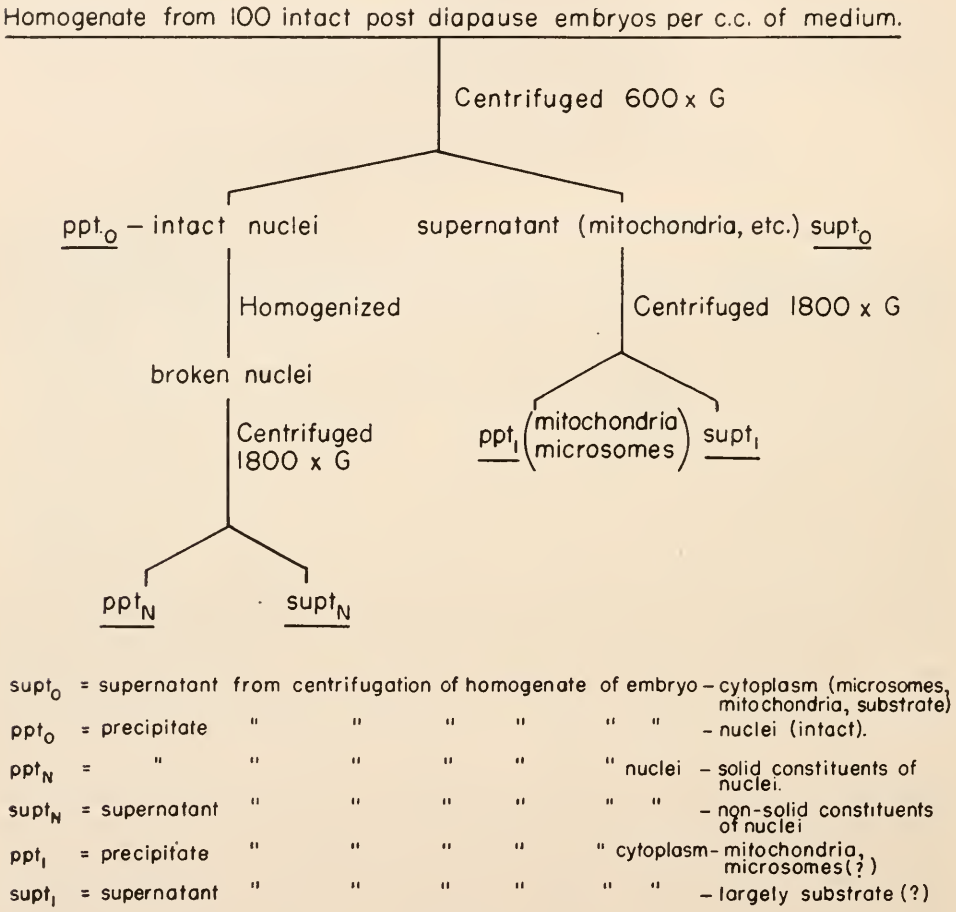
Homogenate from 100 intact post diapause embryos per c.c. of medium.
Centrifuged 600 x G
ppt$_0$ – intact nuclei
supernatant (mitochondria, etc.) supt$_0$
Homogenized
Centrifuged 1800 x G
broken nuclei
Centrifuged 1800 x G
ppt$_1$ (mitochondria microsomes)
supt$_1$
ppt$_N$
supt$_N$
supt$_0$ = supernatant from centrifugation of homogenate of embryo – cytoplasm (microsomes, mitochondria, substrate)
ppt$_0$ = precipitate " " " " " " – nuclei (intact).
ppt$_N$ = " " " " " " nuclei – solid constituents of nuclei.
supt$_N$ = supernatant " " " " " " – non-solid constituents of nuclei
ppt$_1$ = precipitate " " " " " cytoplasm – mitochondria, microsomes(?)
supt$_1$ = supernatant " " " " " " – largely substrate (?)

FIGURE 1.

MATERIALS AND METHODS

Embryos of the grasshopper (*Melanoplus differentialis*), dissected from eggs of known developmental and temperature history, have been used throughout, while all methods employed were essentially similar to those already described (Bodine and Lu, 1950a). Oxygen uptake was measured with standard Warburg manometers at 25° C. using flasks of 5 ml. capacity. Micro-differential manometers were used for isolated nuclei and nuclear homogenates. Preparation of homogenates and

fractionation of cellular parts were carried out as previously indicated (see Fig. 1) (Bodine and Lu, 1950a). Cytological examinations, using the phase microscope, were made on all materials used. Ringer's solution, phosphate buffered (pH 6.8), was used as suspension medium. Concentrations of intracellular constituents were so chosen that 1 ml. of medium contained the equivalent of those obtained from 100 embryos of known developmental history. Figure 1 gives a diagramatic outline of procedures as well as symbols for fractions produced.

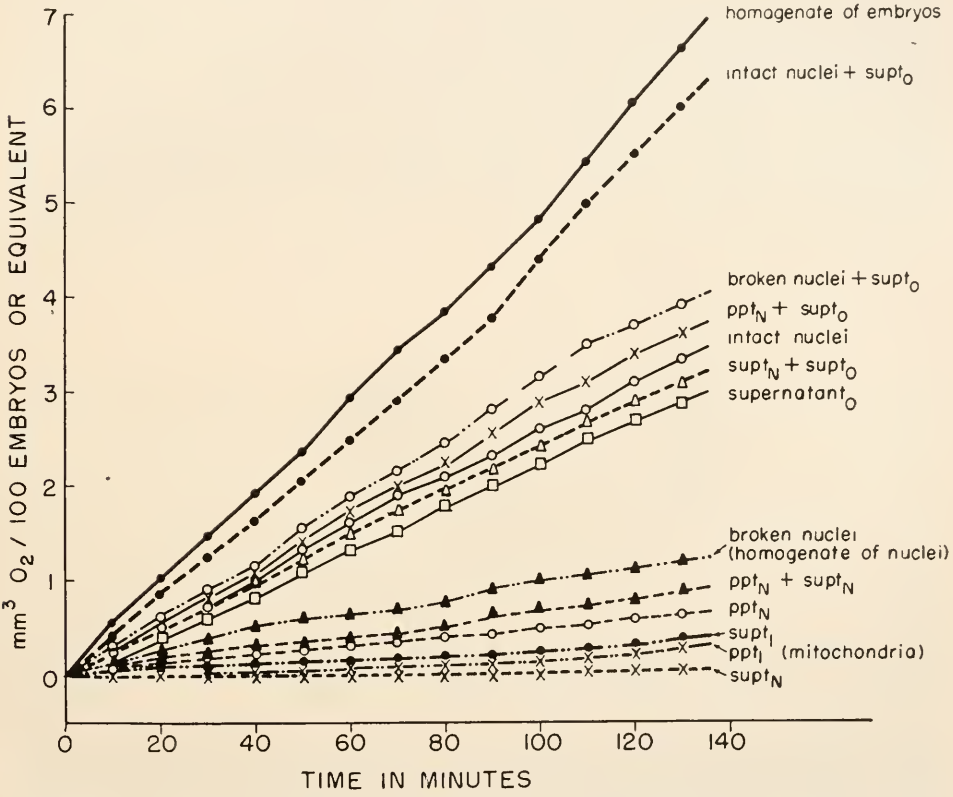

FIGURE 2. Shows oxygen uptake of homogenate of embryo and its constituent parts. Ordinate, mm.^{3} oxygen per 100 embryos or its equivalent. Abscissa, time in minutes. Symbols same as in Figure 1.

RESULTS OF EXPERIMENTS

Inasmuch as results for different lots of embryos are qualitatively similar, only typical cases for eggs of uniform ages and developmental histories will be presented. In general, all embryos and their products thus far tested have given similar results.

The relations between the oxygen uptake of mitotically active (postdiapause) and blocked (diapause) embryos and their homogenates have previously been pointed out (Bodine, 1950). We shall deal here with the endogenous oxygen uptake of embryo homogenates, nuclei, and nuclear homogenates, as well as specific

differences and similarities between enzyme-substrates in the various intracellular parts. Results for typical experiments are graphically shown in Figures 2 and 3.

An examination of Figure 2 shows that the endogenous oxygen uptake of fractionally separated intact nuclei and supernatant (cytoplasm) is approximately 50 per cent of that for the homogenate from which they are derived (Bodine and Lu, 1950a). Upon recombination of the two parts ($ppt._o$ + $Supt._o$) oxygen uptake is approximately 90–95 per cent of that for the original homogenate. These results confirm those previously reported for somewhat similar experiments (Bodine and Lu, 1950a). The intact nuclei apparently have their own enzyme-substrate systems which seem quite distinct from those of the cytoplasm. The cytoplasm, on the other hand, contains both enzymes (mitochondria, microsomes) and substrates (Bodine and Lu, 1950b). Washed and concentrated mitochondria and microsomes respire little if at all and the same is true for the cytoplasmic supernatant after their removal from it (Figs. 2 and 3). Since washed intact nuclei can be broken by homogenization (Bodine and Lu, 1950b), it becomes of some interest to compare

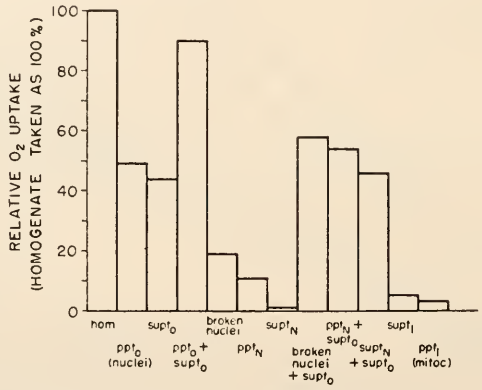

FIGURE 3. Shows relative oxygen uptake for 120 minutes in terms of homogenate of embryo as 100 per cent. Symbols as in Figure 1.

oxygen uptake of intact nuclei with that of their constituent parts and also to determine, if possible, something of the relative specificity of enzyme-substrates of nuclei and cytoplasm. Oxygen uptake of broken nuclei (homogenate) is reduced below that of the intact ones and falls to approximately 30–35 per cent of the original value (Figs. 2 and 3). The broken nuclei are readily separated by fractional centrifugation into solid particles and a liquid supernatant. The oxygen uptake of the supernatant nuclear fluid is practically zero while the solid fraction, even though washed, respires at approximately 50 per cent of the nuclear homogenate rate (Figs. 2 and 3). By recombining the nuclear fragments with the fluid supernatant, a close approximation to the original oxygen uptake is obtained, similar to the case of recombinations of the constituent parts of embryonic homogenate (Bodine and Lu, 1950b).

By making suitable combinations of nuclear and cytoplasmic fractions, rather striking evidence for a marked degree of specificity in the endogenous oxygen enzyme-substrate systems becomes apparent. Broken nuclei (or homogenates of

nuclei) added to cytoplasm produce a greater oxygen uptake than cytoplasmic or nuclear homogenates alone and the sum total of the oxygen uptake is approximately equal to the sum of that for the two systems (Fig. 3). Nuclear supernatant added to cytoplasm produces no significant change in the oxygen uptake over that for cytoplasm alone. The solid particles of the nuclei added to cytoplasm produce an oxygen uptake equal to that of the sum of the two constituent fractions. It seems reasonable to assume, therefore, that the enzyme activity of the nucleus is confined to the solid parts and that substrate resides largely in the fluid parts. Since cytoplasm produces no marked increases in oxygen uptake when added to nuclear fluids, it would seem that mitochondria and other enzyme-bearing structures in cytoplasm do not act upon nuclear substrates and that a degree of specificity exists for cytoplasm and nucleus as regards this type of reaction (Figs. 2 and 3). Inasmuch as endogenous oxygen consumption is that confined *entirely* to the inherent enzyme-substrate systems of the cell, it is not possible from the above results to separate or point out specific types of enzymes involved in these reactions, but it does seem reasonable to assume that some degree of specificity does exist for the nuclear and cytoplasmic respiration systems.

Discussion

The extent to which a specificity for the enzyme-substrate systems of the cytoplasm and nucleus can be demonstrated should throw some light upon the probable chemical interchanges between these structures in the normal intact cell. Evidence gained from the localization of certain chemical compounds like the nucleic acid derivatives would seem to indicate but little passage of materials between the two parts (Caspersson, 1950). That some substances must constantly interchange between the two structures seems reasonable to suppose in view of the equilibrium normally existing between them (Caspersson, 1950; DeRobertis, Nowinski and Saez, 1950). From the present observations on the embryonic cells of the grasshopper, it would seem that for the endogenous oxygen uptake studied, the two systems are more or less distinct. Such a postulation would hold only for those enzyme-substrate systems connected with the endogenous oxygen uptake and would in no way contribute information as to the parts played by other systems. Since the living cell of the grasshopper embryo is stimulated by 2,4-dinitrophenol only when *intact,* while methylene blue stimulates oxygen uptake almost solely by its action on the intact nucleus, one might reasonably assume from such reactions a marked difference in the physico-chemical workings of the various parts of the cell (Bodine and Lu, 1950c). Evidence recently acquired shows that succinate added to homogenates of embryo increases oxygen uptake while no effect is noted when intact or homogenized nuclei are employed. Structural or morphological relations and "intactness" of cells undoubtedly contribute much to the rates at which various localized enzyme-substrate systems work. Localization of substrates in the intact cell is greatly changed or disturbed in homogenates. However, in the case of the embryo, isolated intact nuclei—when combined with cytoplasm—show oxygen uptake of approximately 90–95 per cent of that found for the homogenate from which the constituents were taken (Bodine and Lu, 1950b).

Summary

1. The endogenous oxygen uptake of the homogenate of the embryo of the grasshopper, *Melanoplus differentialis,* along with the intracellular constituents of the embryonic cells has been measured.

2. The endogenous oxygen uptake of intact washed nuclei and their homogenate as well as that of the intranuclear constituents has also been measured.

3. Respiratory enzymes of the nucleus are located in the solid particles obtained from fractional centrifugation.

4. Enzymes of cytoplasm and nucleus appear specific since no change in oxygen uptake occurs when they are added to reciprocal substrates.

5. Fractions of homogenates of embryo or nuclei when recombined have oxygen uptakes of approximately 90–95 per cent of the original homogenates.

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CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENT OF SQUID (LOLIGO PEALII) NERVOUS TISSUE

S. J. COOPERSTEIN AND ARNOLD LAZAROW

Western Reserve University School of Medicine and Marine Biological Laboratory, Woods Hole, Mass.

In the course of investigations designed to elucidate the metabolic differences between normal and chromatolytic nervous tissue, the distribution of succinic dehydrogenase and cytochrome oxidase was studied in various parts of the nervous system of the dog. We found that whereas white matter and spinal nerve had measurable quantities of cytochrome oxidase, both of these tissues contained less succinic dehydrogenase than could be detected by the methods used (Cooperstein, Lazarow and Kurfess, 1950; Schneider and Potter, 1943). In contrast, grey matter and spinal ganglia contain measurable quantities of both of these enzymes and the ratio of cytochrome oxidase to succinic dehydrogenase was found to be 3.9 and 3.2 respectively. If this same enzyme ratio were also present in white matter (or in nerve), the succinic dehydrogenase would have been easily detected. Conversely, considering the sensitivity of the succinic dehydrogenase method employed, it is safe to say that the cytochrome oxidase: succinic dehydrogenase ratio is at least 19.0 in white matter and 9.0 in spinal nerve (Cooperstein, 1951).

Since cytochrome oxidase and succinic dehydrogenase are almost invariably present together in tissues, this dissociation of enzyme activity in nerve and white matter warrants further investigation. It therefore seemed advisable to determine the enzyme content in samples of nerve axoplasm which are not contaminated with the myelin sheath. This can be accomplished in the squid (*Loligo pealii*), where the giant axone can be dissected and samples of pure axoplasm can be obtained in amounts sufficient for microanalyses.

Nachmanson *et al.* (Nachmanson and Meyerhof, 1941; Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) previously studied the distribution of a number of enzymes in squid nervous tissue and found that whereas cholinesterase was more concentrated in the nerve sheath, succinic dehydrogenase and cytochrome oxidase were more concentrated in the axoplasm. Since they used macro-analytical methods, combined samples obtained from different axones were required for each determination. Therefore, relatively few determinations were carried out, and some of the activities reported were at the lower limits of the sensitivity of the methods employed. Furthermore, the assay methods used are less dependable than some of the more recently developed techniques (see Discussion). It therefore seemed advisable to repeat this study using micro-methods (Cooperstein, Lazarow and Kurfess, 1950; Cooperstein and Lazarow, 1951).

Experimental

Methods

Dissection: Squid (*Loligo pealii*) were decapitated and the mantle opened along the mid-ventral line. The bilateral nerve trunks originating from each stel-

late ganglion and containing the giant axone were identified. The giant axone was dissected *in situ* and freed of the extraneous connective tissue sheath and of the small axones which surround the giant axone. The axoplasm is surrounded by a single layer of cells, the inner protoplasmic sheath (which corresponds to the vertebrate Schwann cells), and external to this there is a metatropic sheath which is lipoid-containing. This metatropic sheath, which is similar to the vertebrate myelin sheath except that it lies outside the protoplasmic cell layer, is a few microns thick and can be detected in polarized light (Bear, Schmitt and Young, 1937). It blends with the innermost layers of the outer connective tissue sheath which surrounds the axone, and it usually remains attached to the cleaned giant axone. The right cleaned giant axone with surrounding metatropic sheath was removed. A sample of the fibrous connective tissue sheath (plus the many small axones) which surrounds the giant axone was also taken. The left giant axone was similarly dissected, tied at two places (to prevent loss of axoplasm), removed, and placed on a sheet of Parafilm. One of the ligatures was then removed and a sample of axoplasm obtained by squeezing the nerve between two sheets of Parafilm. Pieces of fin nerve, stellate ganglion, and muscle were also taken. The various samples of tissue weighing between 2 and 10 mg. were placed between two folds of a Parafilm sheet and weighed to 1/100 of a milligram on a Roller-Smith Torsion Balance.[1] The tissue was then transferred to a micro conical-tipped glass homogenizer and kept at 0° C. The Parafilm sheets were then re-weighed. One axone yielded sufficient axoplasm for one set of determinations.

Enzyme determinations: In the succinc dehydrogenase assay (Cooperstein, Lazarow and Kurfess, 1950), the tissues were homogenized at 0° C. in a succinate-phosphate solution (0.5 *M* sodium succinate in 0.2 *M* phosphate buffer, pH 7.4) and diluted with the same solution. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferricytochrome *c*]/minute for a final tissue dilution of 1 : 100.[2]

In the cytochrome oxidase assay (Cooperstein and Lazarow, 1951), the tissues were homogenized at 0° C. and diluted with 0.033 *M* phosphate buffer, pH 7.4. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferrocytochrome *c*]/min. for a final tissue dilution of 1 : 100.[3] Two determinations were run on each sample of tissue. In the succinic dehydrogenase assay, 10 μl. and 20 μl. of an appropriate dilution were tested; in the case of the cytochrome oxidase, 2 μl. and 4 μl. were used.

[1] The Parafilm is obtainable from the Marathon Corporation, Menasha, Wisconsin. Suitable weighing papers measuring 10 × 20 millimeters, and weighing between 5 and 10 milligrams are made by stretching the Parafilm sheet at right angles to roll. Tissues enclosed between two folds of Parafilm do not dry out. The Roller-Smith Balance has a total capacity of 25 mg.

[2] Example: When 10 μl. of a 1 : 60 dilution of stellate ganglion were added to 290 μl. of a cytochrome *c*-cyanide solution (final dilution = 1 : 1800) and the logarithms of the extinctions at 550 mμ (corrected for the blank) were plotted against time, the Δ log [cytochrome c^{+++}]/minute was found to be 0.37. This gave a

$$\text{standard enzyme activity} = \frac{\Delta\log[\text{cyto. } c^{+++}]/\text{minute} \times \text{tissue dilution} \times 300}{\text{volume of enzyme added for test} \times 100}$$

$$= \frac{.037 \times 60 \times 300}{10 \times 100} = 0.666.$$

[3] Calculated as in footnote 2.

Results

Succinic dehydrogenase: The succinic dehydrogenase contents of the various tissues are shown in Table I. Stellate ganglion was found to have a significantly

TABLE I

Distribution of succinic dehydrogenase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:1800	8	0.682	0.081	< .001
Muscle	1:1200	7	0.466	0.051	< .001
Cleaned giant axone	1:900	6	0.360	0.033	>0.1
Fin nerve	1:900	5	0.340	0.072	>0.1
Fibrous sheath+small nerves	1:900	7	0.337	0.063	>0.1
Axoplasm	1:900	7	0.324	0.060	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviations from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma \text{ Difference}}$.

higher content of this enzyme than muscle. Although the succinic dehydrogenase contents of the giant axone, fin nerve, fibrous sheath plus small nerves, and axoplasm do not differ significantly from each other, they are all less active than muscle.

TABLE II

Distribution of cytochrome oxidase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:15,000	8	6.46	0.827	< .001
Muscle	1:12,000	8	4.60	0.615	< .001
Fibrous sheath+small nerves	1:3,000	8	1.23	0.090	>0.1
Axoplasm	1:3,000	8	1.19	0.012	>0.1
Cleaned giant axone	1:3,000	7	1.12	0.210	>0.1
Fin nerve	1:3,000	4	1.08	0.105	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviation from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma \text{ Difference}}$.

Cytochrome oxidase: Table II shows the distribution of cytochrome oxidase. The tissues were found to have the same relative order of activity with respect to cytochrome oxidase as they do with respect to succinic dehydrogenase.

Ratio cytochrome oxidase/succinic dehydrogenase: Comparing the ratios of cytochrome oxidase to succinic dehydrogenase for the various tissues, it was found that those tissues with the highest enzyme activities also have the highest oxidase to dehydrogenase ratio (ganglion, 9.48; muscle, 9.88), whereas those tissues with the lowest enzyme activities also have the lowest oxidase to dehydrogenase ratio (cleaned giant axone 3.12; axoplasm, 3.67; fibrous sheath, plus small nerves, 3.65; fin nerve, 3.48).

Discussion

Our results do not agree quantitatively with those obtained by Nachmanson *et al.* (Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) in that we did not find succinic dehydrogenase or cytochrome oxidase to be concentrated in the axoplasm. (However, these findings do not alter the primary conclusions of these authors in that the majority of the succinic dehydrogenase and the cytochrome oxidase of squid nerve is present in the axoplasm since the major fraction of the giant axone is axoplasm.) We found that the fibrous connective tissue sheath (plus small nerves) surrounding the giant axone contains as much enzyme as does the axoplasm. We did not determine the enzyme contents of the axonal membrane plus metatropic sheath (residual tissue after squeezing out the cleaned axoplasm) because it is difficult to get sufficient material for analysis. For example, a cleaned giant axone which gave 2.07 mg. of axoplasm yielded only 0.25 mg. of residual tissue (after extrusion of axoplasm).

It is possible that part of the apparent enzyme concentration which they reported for axoplasm may have been due to drying. In our studies we have taken precautions to prevent drying by enclosing the pieces of tissue between two sheets of Parafilm (the weight loss under these conditions is less than 0.05 mg. in two hours), and less than 15 minutes elapsed between removal of the axone and its homogenization.

While the discrepancy in the results is not completely explainable, our methods are probably more reliable than those used by Nachmanson *et al.* Since they report that the respiratory rate of these tissues decreases progressively with time after removal from the animal, the assay methods which we have employed offer the advantage that much less time is required for completion.

In the case of the cytochrome oxidase assay, they used p-phenylenediamine as a substrate. This method has certain drawbacks which interfere with its accuracy (Rosenthal, 1937; Stotz and Hastings, 1937), and these errors are magnified at low oxygen uptakes. Another factor to consider is the possible toxic action of some of the end products of p-phenylenediamine oxidation (Rosenthal, 1937). The method which we used for cytochrome oxidase is not subject to these limitations since cytochrome *c* itself is employed as a substrate.

Their succinic dehydrogenase determinations were carried out in two ways. In the first, the oxygen uptake was measured in the presence of succinate and cytochrome *c*, the activity being a measure of the complete succinoxidase system which includes cytochrome oxidase, cytochrome *c*, succinic dehydrogenase, and probably other unknown factors. The second method utilizes ferricyanide as a direct electron acceptor from succinic dehydrogenase without the intervention of the cytochromes. Although Nachmanson *et al.* report good agreement for the rate of utilization of

succinate as determined by these two methods, they made their calculations on the assumption that oxygen is reduced to hydrogen peroxide by the complete succinoxidase system. If, however, one recalculates the data on the basis that water is the end product, and if the oxidation does not proceed further than fumarate or malate, views which are more generally accepted (Hogness, 1939; LuValle and Goddard, 1948; Keilin and Hartree, 1940), their two methods do not agree and one reaches the conclusion that the rate of oxidation of succinate by the complete succinoxidase system is twice as rapid as its rate of oxidation by one of the components of the succinoxidase system, *i.e.,* succinic dehydrogenase. This is an obvious impossibility, since the rate of oxidation by the complete system can be no faster than the turnover rate of its slowest component. Thus, the turnover rate of the dehydrogenase as measured in the ferricyanide test must be considerably lower than in the natural succinoxidase system. Similar inhibition by another artificial carrier (methylene blue) used in a test for succinic dehydrogenase has also been observed (Potter, 1941; Cooperstein, Lazarow and Kurfess, 1950).[4] The method which we used for the determination of succinic dehydrogenase has the advantage over the ferricyanide test in that the natural substrate, *i.e.,* oxidized cytochrome *c* is used. The advantages of this assay method over that using the complete succinoxidase system as a test for succinic dehydrogenase have been previously discussed.

The enzyme dissociation previously observed in the white matter and spinal nerve of the dog has not been found in squid nerve or samples of squid axoplasm. In fact, there is a lower ratio of cytochrome oxidase: succinic dehydrogenase in squid axoplasm and sheaths than there is in squid stellate ganglion or muscle. Therefore, the low succinic dehydrogenase content observed in dog nerve is not a general phenomenon. It is possible that the large amount of myelin present in this species somehow interferes with the determination of succinic dehydrogenase. However, since the addition of nerve or white matter to other tissues does not result in any inhibition of their succinic dehydrogenase activity, this explanation would not appear to account for the results.

Summary

1. The cytochrome oxidase and succinic dehydrogenase contents of various squid tissues, including giant axone and samples of axoplasm, have been determined and compared with previous findings in dogs. In the case of both enzymes studied, the stellate ganglion of the squid is approximately 1.5 times as active as squid muscle. Squid ganglion has approximately six times as much cytochrome oxidase as the other nervous tissues examined, but only about two times as much succinic dehydrogenase. Our findings, in contrast to previously reported results, reveal no evidence of concentration of these enzymes in axoplasm.

2. The markedly low (or absent) succinic dehydrogenase content which was found in dog nerve and white matter was not observed in the case of the squid nerve, axoplasm, or sheaths.

[4] It has been further reported that a ferricyanide concentration of approximately 1/50th that used in the assay method for succinic dehydrogenese causes a 28 per cent inhibition of the oxygen uptake of the complete succinoxidase system (Barron and Singer, 1945). We have found that a concentration of ferricyanide equal to that used in the assay method causes a 91 per cent inhibition of rat brain succinoxidase.

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PHOSPHATASE ACTIVITY IN TETRAHYMENA [1]

ALFRED M. ELLIOTT AND ROBERT L. HUNTER [2]

Department of Zoology, University of Michigan, and Marine Biological Laboratory, Woods Hole, Massachusetts

Histochemical studies by Weisz (1949) with normal and re-organizing Stentors and those by Sullivan (1950) with *Colpidium campylum* have demonstrated the presence of phosphatases in Protozoa. The present investigation was undertaken to increase our knowledge concerning the action of these enzymes in the ciliate protozoan, *Tetrahymena geleii,* and to correlate this activity with growth.

Materials and Methods

The organism employed in this investigation, strain E, was maintained in 250 ml. Erlenmeyer flasks under aseptic conditions except where sterility was not necessary. The media were prepared with double glass-distilled water and were sterilized in the autoclave 10 minutes at 15 pounds pressure. The pH was adjusted to 7.4 with 1/*N* NaOH before sterilization except where pH was specifically under investigation. All cultures were incubated at 24° in the dark. The numbers of organisms were estimated with the Klett-Summerson photoelectric colorimeter according to a method reported earlier (Elliott, 1949b).

Analyses for total and inorganic phosphorus were performed with the methods of Fiske and Subbarow (1925). At stated intervals 6-ml. samples were withdrawn aseptically with pipettes and the organisms separated from the supernatant by centrifugation (100 times gravity for 2 minutes). Phosphorus determinations were made on 2-ml. aliquots of the supernatant. The organisms were washed twice and then re-suspended in distilled water for estimating numbers in the colorimeter. Finally, where indicated, total phosphorus determinations were made on 2-ml. aliquots of the organisms. All experiments were repeated several times and the most representative selected for reporting here.

Experimental

Presence of phosphatases

In order to demonstrate the presence of phosphatases (nucleotidases) in Tetrahymena, a heavy suspension of the cells (approximately 20,000 cells/ml.), taken from proteose-peptone medium during the logarithmic growth phase, was washed twice in distilled water and then suspended in a 0.0005 *M* solution of the nucleotide,

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies. The authors are grateful to Merck and Company for supplying the amino acids and vitamins used in this investigation, and to Dr. E. L. R. Stockstad of the Lederle Laboratories for furnishing the protogen, without which these experiments could not have been carried out.

[2] Teaching Fellow, Department of Zoology, University of Michigan.

adenylic acid. For convenience the rigid sterile technic used in the remainder of the experiments was somewhat relaxed because it was felt that the combined effects of deficient medium and bacterial predation by the ciliate were adequate to control the effect caused by any slight contamination that might occur. The pH was adjusted electrometrically (Beckman) to 7.4 with 1/*N* NaOH. Final pH determinations indicated a slight drop, but not below 7.1. Inorganic phosphorus determinations were made at intervals for 50 hours of incubation and are recorded in Figure 1.

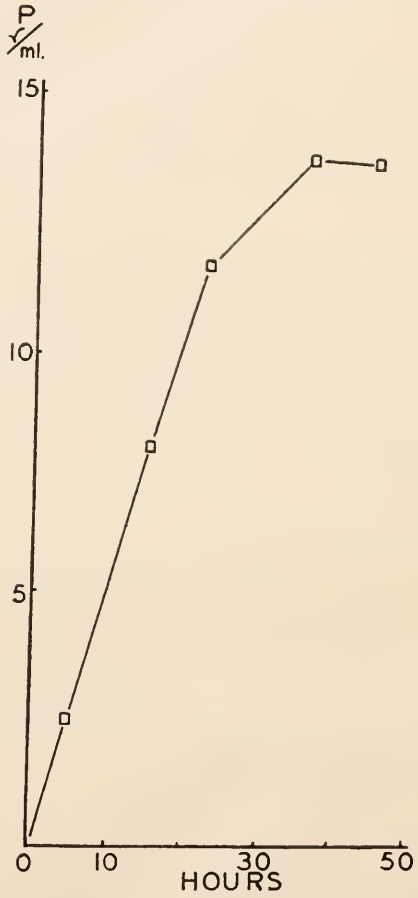

FIGURE 1. Amount of phosphate released plotted against time.

Over 80 per cent of the phosphate in adenylic acid was split off in the first 22 hours and the remaining 20 per cent was released in the next 13 hours. Parallel experiments with cytidylic acid demonstrated similar phosphate release, thus indicating the presence of active nucleotidases.

Location of the enzymes

The question as to whether the enzymes were confined to the cells or released into the surrounding medium was solved by repeating the above experiment, except

that after 14 hours of incubation one-half of the culture was filtered through a Seitz in order to remove the cells from that half; the amount of phosphate release was followed from that time in both flasks. The results are recorded in Figure 2. It is clear that as soon as the cells were removed from the culture, enzyme activity ceased. The enzymes are, therefore, confined to the cells and are not perceptibly released into the culture medium.

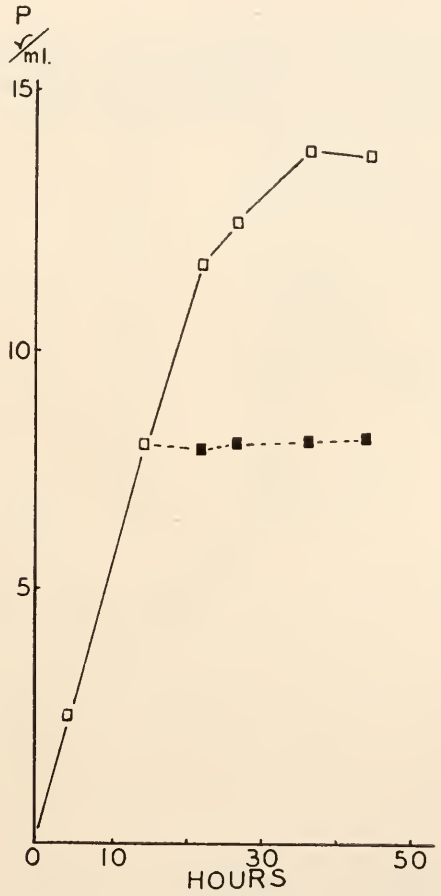

FIGURE 2. Same as Figure 1, except the flask was divided after 14 hours of incubation. Phosphate release indicated by black blocks.

Whether or not the nucleotide was taken into the cell and the phosphate released after being split intracellularly was the next point of interest. If the fate of the adenosine, which results from splitting phosphate from adenylic acid, could be followed, a still more precise location of the enzyme could be predicted. In order to check this point adenylic acid was again prepared as in the two preceding experiments, but this time the absorption of adenine at 260 mμ was followed through 48 hours of incubation, using a Beckman ultraviolet spectrophotometer. Five-ml. samples were removed from the flask at 6–12 hour intervals and the organisms

separated by centrifugation. Appropriate dilutions of the supernatant were made with $N/20$ NaOH and absorption determinations taken.[3] The results of this study indicated that the adenine level remained constant throughout the period of nucleotide breakdown. Apparently, then, the enzyme action takes place either within the cell or at the membrane and perhaps the value of this reaction is to obtain energy for cellular metabolism.

Relation to pH

The activity of the enzymes in relation to pH was demonstrated by following the inorganic phosphate release from adenylic acid at various pH levels. The results are recorded in Figure 3 where the organisms released the greatest amount

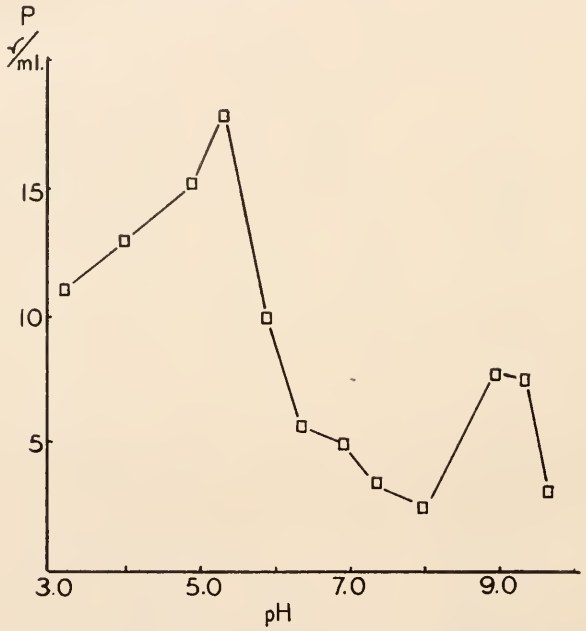

FIGURE 3. Phosphate release at various pH levels.

of phosphate at two levels, namely, 5.3 and 9.2. Since the system is complicated by intact cells in the medium, it is impossible to say that the nucleotidase activity is influenced by pH in the manner shown by these experiments, although it may be.

It might seem strange that all of the experiments were maintained at pH 7.4 rather than at the levels where enzyme activity in this system was optimal. It so happens that both of these levels are outside the range for optimal growth, and therefore might be injurious to the organism. There seems to be no correlation between pH levels which promote optimal phosphatase activity in living cells and those that support optimal growth of those cells.

[3] The adenine determinations were made through the courtesy of Dr. James E. Hogg, Department of Biological Chemistry, University of Michigan. The absorptions of adenine, adenylic acid and adenosine do not differ more than 3 per cent in $N/20$ NaOH solution (Morton, 1942).

Relation to growth

In order to determine phosphatase activity during the logarithmic growth phase of Tetrahymena, it was decided to follow both the release of inorganic phosphate and the phosphate uptake, as indicated by making total phosphorus determinations on both the supernatant and the cells themselves during the logarithmic growth period. The basal medium (Table I) was the same as that employed earlier

TABLE I

Basal medium

	Micrograms per milliliter		Micrograms per milliliter
l-Arginine	600	$FeCl_3 \cdot 6H_2O$	1
l-Histidine	150	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	5
dl-Isoleucine	75	$CuCl_2 \cdot 2H_2O$	5
l-Leucine	75	$MnCl_2 \cdot 4H_2O$	.05
l-Lysine	150	$ZnCl_2$	.05
dl-Methionine	150	Ca pantothenate	.10
dl-Phenylalanine	150	Nicotinamide	.10
dl-Serine	300	Pyridoxine	2.00
dl-Threonine	300	Riboflavin	0.10
l-Tryptophan	150	Pteroyltriglutamic acid	.01
dl-Valine	150	Thiamine	1.0
		Protogen	1.0 unit
Dextrose	1000		
Sodium acetate	1000	Adenylic acid	75
		Guanylic acid	75
$MgSO_4 \cdot 7H_2O$	100	Cytidylic acid	75
$CaCl_2 \cdot 2H_2O$	50	Uracil	75

(Elliott, 1949a, 1950) with the exception that no inorganic phosphate was included in the form of K_2HPO_4 and nucleic acid components were employed instead of yeast nucleic acid. The concentration of these components was raised from 100 to 300 γ/ml., which increased the growth rate by 50 per cent. The only source of inorganic phosphorus was that inadvertently introduced with protogen, an essential growth factor (Stokstad *et al.*, 1949), and this amounted to 2 γ/ml. Six-ml. samples were removed from the experimental flasks and the cells separated by centrifugation; 2 ml. of the supernatant was checked for inorganic phosphorus and another 2 ml. for total phosphorus. The cells were washed twice, then re-suspended in distilled water and numbers estimated in the colorimeter. A 2-ml. aliquot of this suspension was then taken for total phosphorus determinations of the organisms. From these data the curves in Figure 4 were constructed.

The total phosphorus in the supernatant declined at a rate which was the reciprocal of that taken up by the cells, as one might expect. These determinations account for about 95 per cent of the phosphorus. There is an indication that the total supernatant phosphorus continued to decline and the total phosphorus in the cells continued to increase following the growth peak; the reason for this is not clear. It may be that the cells store phosphate after they cease dividing.

The inorganic phosphorus showed the same explosive increase that was noted earlier. Approximately 75 per cent of the inorganic phosphorus is split off while the organisms are still in the lag phase of growth. Once the organisms enter the loga-

rithmic growth phase phosphate is utilized rapidly, hence the curve then shows a decline in the amount of inorganic phosphorus in the medium.

Discussion

Weisz (1949, p. 110), employing histochemical methods, was able to show acid phosphatases in normal Stentors "around the macronuclei, the basal granules of the

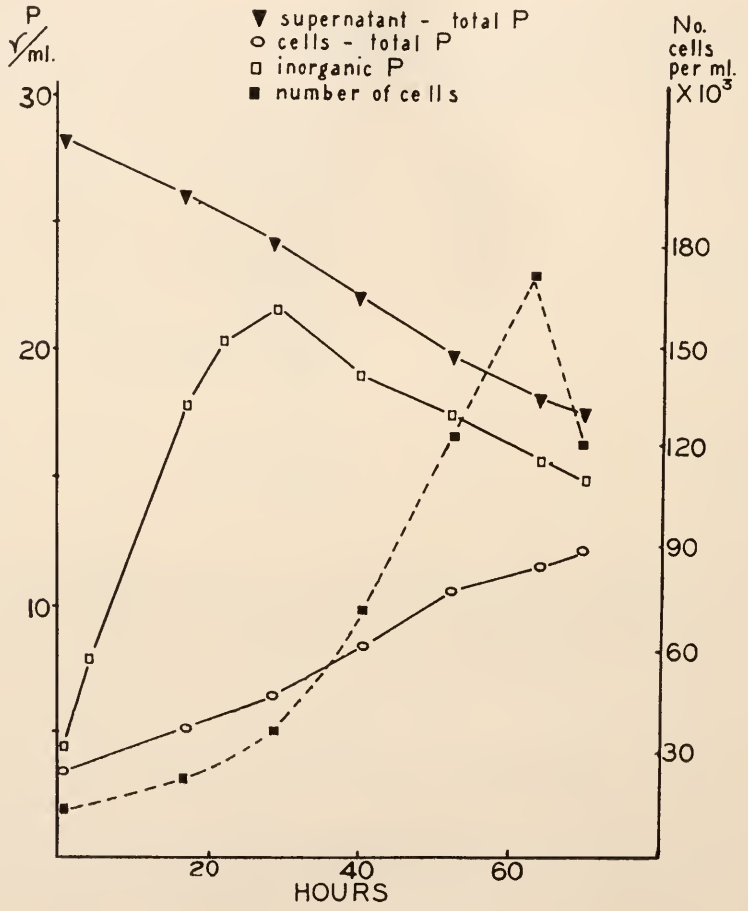

Figure 4. Relation of growth, phosphate release and phosphate uptake.

membranelles and the body cilia, in the endoplasmic fat vacuoles, and around the gastrioles." Sullivan (1950, p. 270), working with similar technics, localized alkaline phosphatase activity "in the perinuclear region of the cell." He used glycerophosphate as a substrate, which may mean that he is not dealing with the same enzymes that are evident in the present investigation. The present observations demonstrate the existence of nucleotidases in *T. geleii* (strain E), but their exact location within the cell cannot be determined from the data. If we are dealing with

the same enzymes as those found by Sullivan, one would expect that the nucleotide (adenylic acid) is absorbed into the perinuclear region of the cell where degradation takes place. This would also mean that the degraded fragments, inorganic phosphate and adenosine, would then be excreted into the medium. However, the present data on both inorganic phosphate and absorption determinations do not demonstrate the absorption of adenylic acid into the cell, only that the molecule is hydrolyzed which may take place at the membrane or intracellularly.

These observations indicate clearly that the enzymes are confined to the organism and are not elaborated into the surrounding medium, which might lend weight to the argument that the enzymes are intracellular. Tetrahymena does produce extracellular enzymes, however, as indicated by its ability to hydrolyze gelatin and casein (Elliott, 1933).

The active nature of these phosphatases in Tetrahymena may have some significance in its natural environment. This organism normally lives at the bottom of ponds where the organic material is undergoing rapid decomposition and where the concentration of inorganic phosphate is high. Numerous studies have shown that the source of this phosphate is in the thin layer of mud at the bottom of the pond or lake where there is a veritable microcosm of flora, fauna and other microorganisms (Einsele, 1938). Perhaps Tetrahymena, along with other microorganisms, aids in splitting off inorganic phosphate from organic debris and helps maintain the phosphate level in these bodies of water.

Summary

1. *Tetrahymena geleii E* possesses phosphatases (nucleotidases) capable of hydrolyzing nucleic acid and its components at a very rapid rate. Eighty per cent of the phosphate in 0.0005 *M* adenylic acid is released in 22 hours when the population level is 20,000 cells/ml.

2. The enzymes are confined to the cells and are not released into the surrounding medium.

3. The cells release phosphate most rapidly at two pH levels, 5.3 and 9.2, being most active in the acid range.

4. Enzyme activity as indicated by phosphate release is greatest in the lag phase of the growth cycle. During the logarithmic growth phase, the released phosphate is taken up by the cells at a uniform rate.

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THE TIDAL RHYTHM OF THE DIATOM HANTZSCHIA AMPHIOXYS

E. FAURÉ-FREMIET

Collège de France, Paris, and Marine Biological Laboratory, Woods Hole, Mass.

Several minute chlorophyll-bearing organisms, which inhabit the sand or the mud of the intertidal zone at high tide, migrate to the surface and show photosynthetic activity induced by the sunlight when the tide is low and the beach exposed. At that time they appear on the beach as irregular green, yellow or brown spots.

This behavior is characteristic not only of the well known metazoan *Convoluta roscoffensis*, but also of certain of the Protophyta. Among these are found the Dinoflagellates of the genera Amphidinium, Gymnodinium, Polykrikos (W. A. Herdman, 1911; Storrow, 1913; Whitehead, 1914; Jorgensen, 1918; E. C. Herdman, 1921; Kofoid and Swezy, 1921; Lebour, 1925), the green flagellates of the genus Euglena (Gard, 1919, 1920; Bracher, 1919, 1929; Carter, 1933; Conrad, 1940), the Chrysomonadian *Chromulina psammobia* (Fauré-Fremiet, 1950b), and several diatoms (Bohn, 1904a, 1904b; Fauvel, 1907; Fauvel and Bohn, 1907).

Under natural conditions, the superficial migration of these protophytic organisms appears to be an expression of an intrinsic rhythm which is synchronized with the tide, and which is complicated by the response to light. This rhythm has been studied experimentally in the laboratory, using the following species: the diatom *Pleurosigma estuari* Smith (Fauvel and Bohn, 1907); the green flagellate *Euglena limosa* Gard (Gard, 1919, 1920; Bracher, 1919, 1929, 1938); the Chrysomonadin *Chromulina psammobia* (Fauré-Fremiet, 1950b).

During the past summer a similar behavior was observed in a population of the diatom *Hantzschia amphioxys* Ehrb.,[1] which was found inhabiting the sand of the beach at Barnstable Harbor, Cape Cod.

During the low tide of mid-day in August, irregular spots of different size were visible on the sandy flat near the experimental park of the Woods Hole Oceanographic Institution at Barnstable Harbor. The surface sand, which is slightly muddy, was removed with a spoon and brought to the laboratory for examination. It was then spread over the bottom of large finger bowls and covered with sea water. These cultures were then exposed to the diffuse light from the window.

On succeeding days (the longest period of observation being 6 days), greenish spots similar to those described above re-appeared on the surface of the sand at the time of the low tide at Barnstable Harbor. These disappeared after two to three hours. A microscopic examination showed that the color was due to the superficial accumulation of a great number of the diatom *Hantzschia amphioxys*, associated with a much less numerous undetermined Gymnodinium, and a Euglena.[2]

Hantzschia amphioxys is of rectangular shape, and is slightly asymmetric, having one face convex and the opposite concave. It varies in size from 60 to 80 micra

[1] I wish to express my thanks to Dr. Paul Conger for the determination of this species.

[2] This Euglena resembles *E. limosa* Gard in the lack of flagella and in the metabolic motion, but differs from that form in possessing small and numerous chromoplasts.

in length, and shows two transverse and radiate chromoplasts which are greenish yellow in color. The sliding motion of this diatom is relatively rapid; they travel in the neighborhood of 400 micra in 10 seconds, or about half their total length in one second.

Individuals isolated from the greenish mass exhibit a positive phototropism. When observed under the microscope in a drop of sea water, they move rapidly to the lighted side. This would explain the superficial dispersion of the diatoms both under natural and experimental conditions.

After a few hours, a similar test shows that the phototropistic sign is reversed, the response then being negative. This being true, it would be expected that the diatoms, in the natural habitat, would move into the shadow of the sand grains, leading to a dissolution of the colored spots. The diatoms then have invaded the deeper sandy layers.

During this photophobic phase, the Hantzschia agglutinate among the sand grains. This may well explain why the rising tide does not wash the diatom population away, and has implications of a great general ecological interest.

The observations on the diatoms made in the laboratory with unwashed sand do not permit an exact appreciation of the end-points in the tidal rhythm. Due to the pale color of *Hantzschia amphioxys*, initial phases in surface accumulation and spreading are difficult if not impossible to recognize, and the same is true of the final stages in the disappearance of the surface masses. The behavior of this diatom presents, therefore, a statistical aspect.

It is certain, however, that this species exhibits a physiological rhythm, which is marked by the periodic reversal of its phototropic sign, and by the agglutinative capacity which characterizes the photophobic state.

This physiological rhythm, when observed *in vitro*, is not directly determined by the periodicity of the tide, although it appears to be synchronized to it. In combination with the direct action of light, it controls the rhythmic behavior of the diatoms under the artificial laboratory conditions, and disappears after a few days.

All these facts are in complete agreement with the results of known experiments on the tidal rhythm of *Euglena limosa*, of *Chromulina psammobia*, and of a ciliate associated with symbiotic Chlorella, namely, *Strombidium oculatum*. In the last case (Fauré-Fremiet, 1948), the living conditions are quite different from those of the sand-dwelling species. The above observations lead to the conclusion that the explanation previously suggested for the behavior of the three forms cited above will also apply in the case of *Hantzschia amphioxys*.

Numerous observations and experiments have been made on the rhythmic behavior of simple physiological events. With respect to those which are found in the Protista, as are those described above, it has been presupposed first, that the physiological rhythm is endogenous, and second, that the periodicity which appears to be exactly defined at the population level, is not necessarily so defined in the individual. In addition, it has been assumed that the approximate period for the complete cycle slightly exceeds the 24 hours of a day.

If this is true in the present instance, each rising tide will cause every individual which, at that moment, is not sheltered and agglutinated in the deep layers, to come out of the sand. In other words, a selective elimination takes place at each flood tide, and hence, by elimination of all the "non-conformist" individuals, the syn-

chronism of the endogenous physiological rhythm with the tidal periodicity is maintained.

When this selective elimination is suppressed, as under continued laboratory conditions, the individual variations in periodicity are asserted, and the resulting progressive overlapping of the endogenous periods leads to the disappearance of the collective rhythm.

The statistical maintenance under natural conditions of a synchronism of physiological rhythm and tide is responsible for the apparent adaptation which assures the permanence of a population of small and moving individuals, in a biotope periodically washed by the sea.

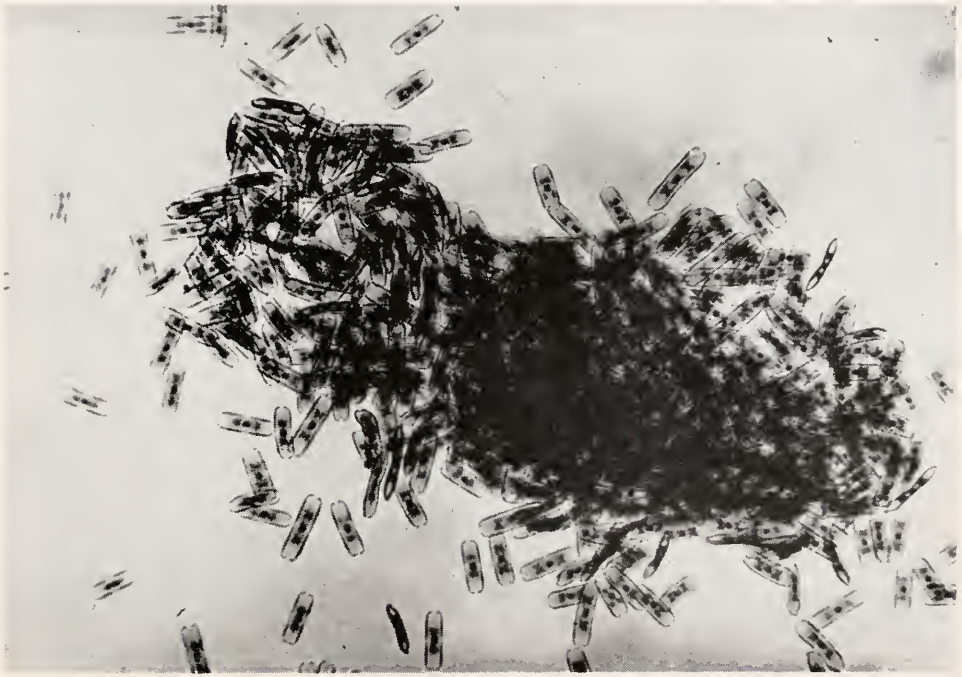

FIGURE 1. A clump of *Hantzschia amphioxys* at the photophobic state, agglutinated on a glass slide under a sand grain layer.

As has been stated above, individuals of *Hantzschia amphioxys* agglutinate to form small clusters in the sub-surface sand when in the photophobic phase. When the same phenomenon is observed in sand on the bottom of a small glass dish or on a microscope slide placed on the bottom of a Petri dish, it may be demonstrated that these clusters adhere to the glass and to the grains of sand. The masses maintain their form and prove to be elastic when they are teased with needles. When fixed while adhering to a slide, it is possible to stain and wash the preparation without freeing the clusters (Fig. 1).

It would appear, therefore, that in the photophobic state, the diatoms are surrounded by a jelly, coagulated by fixative reagents, and possibly a mucoid, which

may be considered as a secretion product. These facts are very suggestive in the light of the common statement that slow coagulation of organic substances plays a role in the flocculation and stabilization of marine sediments.

Many interstitial organisms, such as nematodes, copepods, ciliates, and even the agglutinated diatoms themselves, however, continue to move in the sand. The motion is often sufficiently extensive to result in slight displacements of sand grains which are bound together by diffused organic products. Thus, in spite of a relative fragility, the superficial layers of the sand comprise a relatively stable and coarse framework within which there dwells a permanent interstitial microscopic fauna and flora. This is a fact of some ecological interest.

Marine littoral sands represent peculiar and interesting biotopes with respect to the interstitial microfauna. The comparative study of the sand-inhabiting ciliates of the beaches of Brittany and of Cape Cod (Fauré-Fremiet, 1950a, 1951) shows that some species are closely adapted to the interstitial conditions. When free in the water, or when adjacent surfaces are clean solids, the animals prove to be very fragile. The sand grains of the beach must represent a very complex biotope, for the organic substances which cover them appear to have a protective effect on the unstable cytoplasmic surface of the ciliates. At the same time, the organic substances may cohere to render the "structure" of the superficial sand layers more stable.

It is obvious that the secretory and excretory products of the many sand-inhabiting annelids and molluscs play an important part in the development of sand structure. The above observations show that the same is true for many much smaller organisms; among these are the Protophyta and most certainly the bacteria.

I wish to express my thanks to Dr. L. Hoadley for his kind revision of my manuscript.

Summary

1. A sand-dwelling population of the diatom *Hantzschia amphioxys* Ehrb. is described, which exhibits a tidal rhythm that persists for some days when held without tide under artificial conditions in the laboratory. This rhythm is characterized by a periodic inversion of the phototropistic response, and by an agglutination of the members when in the photophobic state.

2. A comparison of this behavior with that of *Euglena limosa*, *Chromulina psammobia* and *Strombidium oculatum* shows close analogies and suggests that the same explanation may account for the synchronization of physiological rhythm and tide in all cases. It seems to be that the rising tide acts as a selective factor.

3. Organic substances produced by the diatoms contribute to the structural stabilization of the superficial sandy layer, and the presence of this organic material appears to be an ecological condition essential to the interstitial microfauna.

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THE DESOXYRIBONUCLEIC ACID CONTENT OF THE NUCLEUS AS A CYTOTAXONOMIC CHARACTER IN MANTIDS (ORTHOPTERA: MANTOIDEA)

SALLY HUGHES-SCHRADER

Department of Zoology, Columbia University, New York 27

The hypothesis that the nuclear content of desoxyribonucleic acid is a constant character of the species is supported by rapidly accumulating evidence (Boivin, Vendrely and Vendrely, 1948; Vendrely and Vendrely, 1948, 1949, 1950; Mirsky and Ris, 1949; Ris and Mirsky, 1949; Swift, 1950a, 1950b; and others). It moreover appears probable that the higher taxonomic categories will show a characteristic order of magnitude with respect to this value; and polyploid species already tested show the expected stepwise relation. The possibility is thus opened that the amount of DNA per nucleus may prove a useful cytotaxonomic tool in evaluating evolutionary relationships among species whose karyotypes are not analyzable by the methods of comparative cytology. The following report records a preliminary exploration of this possibility for certain problems of karyotype relationship among mantids.

Cytological Background

The first group investigated comprises three species of the taxonomically difficult genus *Liturgousa* of the subfamily Liturgousinae. Closely similar in phenotype and habit, and occupying common or adjacent ranges, they differ widely in chromosome complements (Hughes-Schrader, 1950). *L. maya* has 16 autosomes, while *L. cursor* precisely doubles this number with 32, and *L. actuosa* [1] is intermediate with 22. While the X chromosome appears identical in all three, the autosomes are not morphologically homologous, but show in general an inverse relation of size to number (Figs. 1, 2 and 3). No direct Robertsonian relation (the evolutionary equivalence of one mediokinetic to two acrokinetic chromosomes) is demonstrable between *L. maya* and *L. actuosa*, nor between *L. maya* and *L. cursor*. It is possible, however, that *L. cursor* and *L. actuosa* stand in this relation to each other, if the 10 pairs of apparently rod-shaped autosomes of the former correspond to 5 pairs of V-shaped elements in the latter. No final conclusion is justified since the position of the kinetochore in the short chromosomes of *L. cursor* cannot be established positively. On comparative inspection the chromosomes of the three species appear approximately equal in total mass, but measurement at spermatogonial metaphase shows the total length of the chromosomes to vary inversely with chromosome number (Table I). The total cytological evidence thus fails to make clear the evolutionary relationship of these species. While precluding polyploidy, it suggests

[1] The erroneous name *L. arcuosa* was used for this species by Hughes-Schrader (1950). Its correct designation and description have since then become available in Rehn, 1951, *Trans. Ent. Soc.* **76**: 363–383.

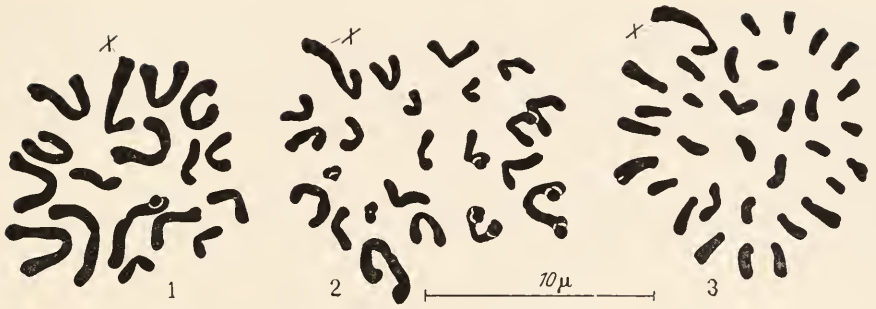

FIGURE 1. Spermatogonial metaphase—*Liturgousa maya.*
FIGURE 2. Same—*Liturgousa actuosa.*
FIGURE 3. Same—*Liturgousa cursor.*

(All drawings made with camera lucida, at table level; Zeiss apochrom. obj. 90, comp. oc. 20; magnification as reproduced is uniform in all and indicated by scale on each. Fixation Sanfelice; stain Feulgen.)

that changes in total amount of chromosome material, as well as changes in its distribution and in number of kinetochores, have been associated with this divergence. Measurement of metaphase chromosomes in any species can, however, at best give but an approximate index of the amount of chromosome material, for differences in intensity of dye and tightness of coiling cannot be adequately appraised. It was therefore hoped that the photometric determination of the relative amount of DNA in the interphase nucleus, by providing a more accurate index of the substance measured in Feulgen-dyed metaphase chromosomes, might elucidate the interrelationship of the *Liturgousa* species.

With the dual purpose, first, of providing a standard of comparison for possible interspecific differences in the nuclear DNA content among the *Liturgousa* species, and second, of ascertaining whether or not cytotaxonomically useful constants of nuclear DNA content characterize different subfamilies, measurements were also made on a second group of mantids from the very distantly related subfamily Manteinae. These were chosen on the basis of close similarity in karyotype, and include

TABLE I

Average total length, in arbitrary units, of metaphase chromosomes

Species	Chromosomes 2n ♂	Spermatogonia	Number measured	Meiosis II	Number measured
Liturgousa maya	16+X	59.9	3		
Liturgousa cursor	32+X	42.6	3		
Liturgousa actuosa	22+X	51.5	3		
Choeradodis rhombicollis	28+X^1X^2Y	56.3	3		
Stagmomantis carolina	24+X^1X^2Y	62.5	4	53.6	4
Stagmomantis heterogamia	24+X^1X^2Y	68.2*		58.6	6
Tauromantis championi	24+X^1X^2Y	66.0	5		
Pseudomiopteryx infuscata	16+X	26.9*		23.1	8

* Calculated from ratio of total length of meiotic to gonial chromosomes in *Stagmomantis carolina.*

Tauromantis championi, Stagmomantis carolina, and *Stagmomantis heterogamia* (Figs. 4, 5 and 7). The chromosome number ($2n♂$) is 27 in each, comprising 12 pairs of autosomes and the compound sex chromosomes X^1, X^2 and Y; the chromosomes are similar in size, with the exception of the Y chromosome, which is identical in the *Stagmomantis* species but considerably larger in *Tauromantis* (Hughes-Schrader, 1950).

Of special interest would be a comparison of DNA nuclear content between *Choeradodis* and the Manteinae. The genus is currently elevated to separate subfamily rank, but *Choeradodis rhombicollis* is shown by the cytological evidence provided by the morphology and behavior of the compound sex chromosomes to stand in closer relation to the X^1X^2Y Manteinae than is recognized taxonomically (Hughes-Schrader, 1950). The autosomes of *Choeradodis,* while numbering two more pairs than are found in the Manteinae, are individually considerably smaller and their total length appears approximately the same as in the latter. Unfortunately, the only

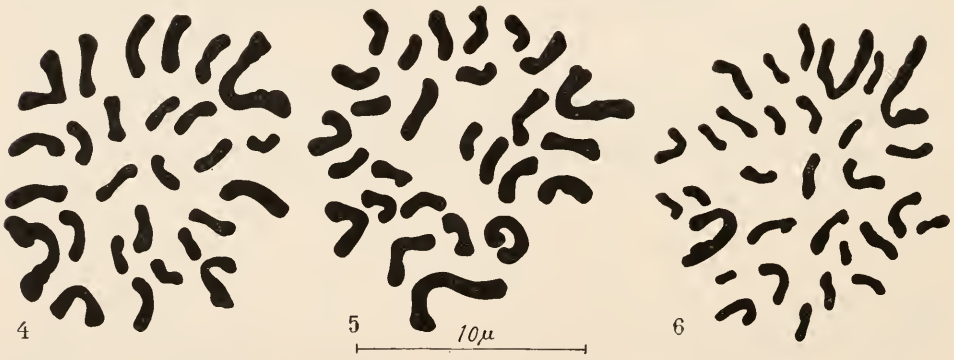

FIGURE 4. Spermatogonial metaphase—*Stagmomantis carolina.*
FIGURE 5. Same—*Tauromantis championi.*
FIGURE 6. Same—*Choeradodis rhombicollis.*

material of *Choeradodis* available for photometry gives evidence of considerable cytological abnormality; the results obtained from it can be regarded as suggestive only.

Finally, measurements were made on nuclei of *Pseudomiopteryx infuscata* of the subfamily Pseudomiopteriginae. Here, while the chromosome number ($2n♂ = 17$) is the same as in *Liturgousa maya,* the total mass of the chromosomes appears to be far less. When compared with the manteine species *Stagmomantis heterogamia,* the total length of the chromosomes of the *Pseudomiopteryx* complement is seen to be less than half that in the first named species (Table I and Figs. 7 and 8). The nuclear DNA content of such a karyotype is of special interest since the distribution of chromosome numbers among all cytologically known mantids shows two peaks—a dominant one at 27 with outlying species ranging upwards to 39, and a secondary peak embracing the 15 to 19 chromosome range. Furthermore, in many of the high-number karyotypes the X chromosome (or its derivative arms in X^1 and X^2) is of extreme length relative to the autosomes and in comparison with the X of certain low-number karyotypes such as that of *Pseudomiopteryx.* Thus, the

possibility exists that polyploidy—its establishment made possible by the stabilization of the sex chromosome mechanism through fusion of the X chromosomes, along lines similar to those first postulated by Bauer (1947) for certain Dermaptera—has been involved in the evolution of the Mantoidea. Of course no implication of any recently established diploid to tetraploid relation between *Pseudomiopteryx* and *Stagmomantis* is intended; taxonomic and cytologic considerations alike preclude it. They were chosen, from the limited material available, as roughly representative in general features of chromosomal mass, number, and morphology of the postulated ancestral karyotypes.

Material and Methods

The material used comprises testes from males of the 8 species enumerated above; collection and field notes have been recorded previously (Hughes-Schrader,

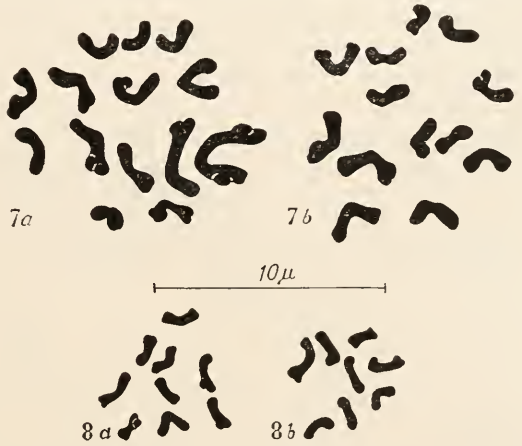

Figure 7. Second meiotic metaphase—*Stagmomantis heterogamia*. a. Secondary spermatocyte with X^1 and X^2; b. Secondary spermatocyte with Y.
Figure 8. Second meiotic metaphase—*Pseudomiopteryx infuscata*. a. Secondary spermatocyte with X; b. Secondary spermatocyte with no X.

1950). For photometric measurements two individuals of each of the three species of *Liturgousa,* and one of each of the other 5 species, were used. The material was fixed in Sanfelice, washed in water overnight, embedded in paraffin and sectioned at 8 or 10 μ. Sections from each of the *Liturgousa* species were mounted together on consecutive slides, as were those of the Manteinae-*Choeradodis* group. In addition the slides of the latter series carried sections from one testis of the first group to provide a standard for comparison of data from different slides. The limited material available restricted direct comparison of *Pseudomiopteryx* to *Stagmomantis heterogamia* and *Choeradodis* of the second group.

Staining was by the Feulgen method; alternate slides in each series constituted unhydrolyzed controls. The photometric measurements were made by the Pollister and Ris (1947) method and with the apparatus described by Swift (1950a). DNA in the nucleus was measured as the extinction of the Feulgen dye at the 546 mμ line, isolated by a Farrand Interference Filter No. 2756 from an AH4 mercury

vapor lamp. The relative amount of DNA per nucleus was calculated in arbitrary units of Feulgen dye, according to the formulae given by Swift (1950a): the measured extinction of a cylinder 3.8 μ in diameter through an uncut nucleus is multiplied by the squared radius of the cylinder and divided by the percentage of the total nuclear volume represented by the cylinder. The nuclei measured deviated only slightly if at all from the spherical; major and minor axes were measured and the mean considered as the diameter. Feulgen preparations following Sanfelice fixation of mantid testes often show, in control and test slide alike, a diffuse stain in the cytoplasm. Since the intensity of this cytoplasmic dye varied significantly among some of the species tested, the extinction of each nucleus measured was corrected by a factor relating the amount of overlying cytoplasm to the cytoplasmic extinction.

Spermatid nuclei were chosen for measurement since they may be expected to be relatively free of variation in DNA associated either with mitosis or with special metabolic function. Moreover, throughout the series of species tested, spermatid nuclei proved highly comparable morphologically. Fortunately also, in all these species, the chromosomes continue to de-spiralize after the second meiotic telophase and, just prior to the elongation of the nucleus to form the head of the sperm, they reach a state of relatively extreme extension and rather diffuse staining which is favorable for photometric measurements. To avoid variation stemming from differential penetration effects, only nuclei from cysts at or very close to the surface of the testis were utilized. All testes used, with the exception of the *Choeradodis* material, are from nymphal males and provide an abundance of uniform, normal-appearing spermatid nuclei of the desired stage. The only available material of *Choeradodis* comes from an adult male with spent testes. Only a few cysts of the required stage of spermatid are present; and these give evidence—through wide variation in nuclear size and stain and a high frequency of giant and micro nuclei—of abnormality in chromosome distribution in the preceding mitosis.

To facilitate correlation of the photometric data with the cytological findings, estimates were made of the total length of the chromosomes at spermatogonial or second meiotic metaphase in Feulgen preparations. It was found that a reasonable agreement in values was obtained only from the relatively few plates in which all chromosomes were extended fairly flatly in one plane; the figures on total length in Table I are the mean values from measurements of from three to eight such metaphase plates. The maximum deviation from the average did not exceed 8 per cent, except in *Tauromantis* and *Choeradodis* plates, where it reached 14 per cent. Camera lucida drawings, at 1800 ×, were enlarged four fold by pantograph and the length of each chromosome recorded with a map measure. It proved impossible to gauge the diameter of the chromosome satisfactorily by this method; and since differences in chromosome diameter are distinguishable among these species, the total chromosome length can give only an approximation of total mass.

Results

The photometric measurements of the relative amount of DNA in spermatid nuclei disclose a range of values characteristic for each of the species tested. The results are summarized in Table II, in which each major vertical column contains data from a single slide. For brevity in presentation the results are recorded as the

mean values of the different samples of nuclei measured. The use of the mean DNA per nucleus value as a species constant appears justified for purposes of preliminary interspecific comparison; to what extent the spread of values about the mean reflects real variation among the nuclei, and to what extent errors in method and measurement, is unknown. The pattern of scatter of the values obtained does show the effect of the segregation of the sex chromosomes, especially in the spermatids of the manteine species where two relatively large X chromosomes have segregated from a small Y. When DNA content is plotted against number of nuclei, some indication of a bimodal distribution is apparent in each sample. It is also of interest to note, relative to the variation within the species range, that no nucleus measured contains DNA in an amount equal to or closely approaching twice the mean value, and in only four instances was the maximum value twice that of the minimum obtained.

TABLE II

Average amounts (in arbitrary units of Feulgen dye) of DNA in spermatid nuclei of mantid species. (a,b = 2 individuals tested; N = number of nuclei measured)

Species	Slide 1			Slide 2			Slide 3			Slide 4			Slide 5			Total number of nuclei measured
	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	
Liturgousa maya a	1.11	0.041	14	1.04	0.049	16										30
Liturgousa maya b				1.06	0.026	15										15
Liturgousa cursor a	1.01	0.026	10	0.98	0.027	15										25
Liturgousa cursor b										1.04	0.029	20				20
Liturgousa actuosa a	1.51	0.041	15	1.48	0.049	16	1.50	0.059	12	1.43	0.025	20				63
Liturgousa actuosa b										1.65	0.020	20				20
Choeradodis rhombicollis							1.52	0.086	20				1.18	0.027	36	56
Stagmomantis carolina							1.80	0.073	20							20
Stagmomantis heterogamia							1.65	0.060	20				1.59	0.041	26	46
Tauromantis championi							1.73	0.077	17							17
Pseudomiopteryx infuscata													0.74	0.024	20	20

Before turning to a consideration of the results in relation to the cytological problems presented, one other point of general interest should be emphasized. As shown in Table II (slides 2 and 4), the two individuals of *Liturgousa maya* tested are practically identical in respect to the DNA content of spermatid nuclei; the same is true of the two specimens of *L. cursor*. In *L. actuosa,* however, a significant difference is apparent (Table II, slide 4)—the second individual tested shows a value 15 per cent higher than that of the first. In view of this finding, the number of individuals of each species sampled in the present study must be considered as too low to justify final conclusions as to the limits of the specific range in the DNA value of the spermatid.

DISCUSSION

Liturgousinae

In the three species of *Liturgousa* studied, it is clear (Table II, slides 1 to 4) that the amount of DNA per spermatid nucleus does not vary directly with chromosome number and size. The nuclei (haploid) of *L. maya* with 8 autosomes and

those of *L. cursor* with precisely twice as many show no significant difference in DNA content. This is in agreement with the tentative conclusion based on cytological data (Hughes-Schrader, 1950) that a re-distribution of chromosome material and a change of kinetochore number not involving polyploidy has been associated with the evolutionary divergence of these species. In view of the 1 : 1 ratio in DNA it is, however, surprising to find a ratio of 1.4 : 1 in the total length of metaphase chromosomes between *L. maya* and *L. cursor* (Table I) ; moreover, the *L. maya* chromosomes are appreciably thicker than those of *L. cursor.* This discrepancy may be more apparent than real in respect to the actual amount of Feulgen-positive material present, since considerable differences in intensity of stain and in degree of condensation of the metaphase chromosomes are not detectable by the eye.

But by far the most interesting result of the comparison of the *Liturgousa* species, and one not to be anticipated from the cytological evidence, is the wide divergence in DNA values of *L. actuosa* from its two sister species. Closely similar to *L. maya* and *L. cursor* in phenotype, intermediate between them in chromosome number and size and in total chromosome length, *L. actuosa* is characterized by an amount of DNA per spermatid which is half again as large as theirs. No differential polyteny among the chromosomes is discernible at the mitosis preceding spermatid formation; and in chromosome diameter—admittedly difficult to gauge with accuracy—*L. actuosa* appears to be intermediate between *L. maya* and *L. cursor.* That a change of such magnitude in the relative amount of DNA per nucleus may be effected among closely related species without a corresponding visible change in total chromosomal mass is, aside from its interest relative to chromosome structure, prejudicial to the hope that a DNA species constant will prove cytotaxonomically useful. The significance of this finding is of course dependent on the validity of my assumption that the spermatid nuclei of the species concerned are actually as comparable in respect to the timing of DNA synthesis as they appear to be in all cytologically demonstrable characters. If any synthesis, possibly anticipatory of the next mitosis, takes place in the spermatid nucleus, it may well have undergone interspecific variation in time or rate. It is thus desirable that the DNA content of the spermatid be compared to that of somatic nuclei in each of the species involved; unfortunately, this must await the collection of further material.

Manteinae

Uniformity in spermatid DNA characterizes the three species of the subfamily Manteinae—*Tauromantis championi, Stagmomantis carolina,* and *Stagmomantis heterogamia;* the mean values obtained (Table II, slide 3) do not differ significantly, and in range of values the four samples of nuclei measured are practically identical. This is in harmony with the cytological findings, for these species have the same number of chromosomes and are closely similar in chromosome morphology and behavior. In total length of chromosomes as measured at spermatogonial metaphase also, *Tauromantis* and *Stagmomantis carolina* are in good agreement (Table I). No measurable spermatogonial metaphases are available for *Stagmomantis heterogamia,* but at second meiotic metaphase the total length of its chromosomes agrees, within the probable error of measurement, with that of *S. carolina.* Assuming the same ratio in length between meiotic and gonial chromosomes in the

two *Stagmomantis* species, the total chromosome length at spermatogonial metaphase in the three manteines (Table I) shows no greater variation than is obtained in measurements of different plates in one species.

Relationships among X^1 X^2 Y mantids

The species of the subfamily Manteinae characterized by the compound sex chromosomes—X^1X^2Y ♂—$X^1X^1X^2X^2$ ♀—form a closely linked natural group cytologically—their joint possession of this particular sex chromosome mechanism denotes, with a high degree of probability, their descent from a single ancestral species (White, 1941). Species with a sex trivalent strikingly similar in structure and behavior are now known in four other subfamilies, and the cytological evidence indicates a closer relation to the Manteinae than is expressed in their taxonomic placement (Hughes-Schrader, 1950). Of these non-manteine X^1X^2Y species, material of only one—*Choeradodis rhombicollis*—was available for comparison of DNA values with the Manteinae. And as noted above, the few cysts of spermatids remaining in this adult testis give evidence of abnormalities in chromosome distribution in the preceding mitosis, which precludes a determinaton of the DNA constant of the species. The mean value for DNA in the spermatid in the two disparate samples measured, however, falls below that of the Manteinae (Table II, slides 3 and 5) ; and since not one nucleus in 56 measured exceeded in DNA the maximum value obtained in the Manteinae, we may expect the real value for *Choeradodis* to be somewhat lower than the general manteine level. This is of interest since *Choeradodis* has two more pairs of autosomes than is characteristic for X^1X^2Y Manteinae. The cytological evidence also indicates that the difference in chromosome number implies no increase in chromosome mass, for the total length of the spermatogonial chromosomes is approximately the same (Table I), and their diameter is appreciably less (Figs. 4, 5 and 6), than in the manteine species. As to the major issue, however—the relationship of X^1X^2Y species currently referred to different subfamilies—more normal material of *Choeradodis* than presently available is obviously necessary in order to test the relevancy of photometric data on nuclear DNA.

Polyploidy

Pseudomiopteryx of the subfamily Pseudomiopteriginae was chosen for photometric measurement of nuclear DNA content because, as noted previously, it represents a group of low-number karyotypes among mantids which apparently contain not more than half the Feulgen-positive chromosome material present in many high-number karyotypes such as the three species of the subfamily Manteinae here tested. This in turn suggests that polyploidy may have been involved in the early differentiation of high and low chromosome numbers in ancestral mantid stocks. The photometric data (Table II, slide 5) do indeed support this suggestion: the relative amounts of DNA per spermatid in *Pseudomiopteryx* as compared with *Stagmomantis heterogamia* closely approach a 1 : 2 ratio (ratio = 0.47). A greater disparity is apparent in total length of chromosomes at the last preceding mitosis (Table I) ; the ratio here is 0.39. Since, however, the arms of second meiotic metaphase chromosomes of both species tend to curl at the tips and the resulting error in measurement is proportionately greater in the shorter chromosomes of *Pseudomiopteryx,* this finding need not be considered wholly out of line with the photo-

metric data. On the other hand, the difference in diameter between the chromosomes of *Stagmomantis* and *Pseudomiopteryx,* as shown in Figures 7 and 8, gives the impression of a greater disparity in total mass than is indicated by the ratio in total length.

I am convinced that polyploidy has played a greater role in the evolution of animal karyotypes than was earlier so generally assumed. Nevertheless, the hypothesis that polyploidy was involved in the evolution of the Mantoidea must be advanced with reservations. In the first place, the chromosome numbers now known among mantids embrace nearly all intermediate values between the low and high peaks. Secondly, the photometric data here reported do not exclude the possibility of a differential polyteny in the spermatids of the different species considered. And finally, a change of great magnitude in the DNA content of spermatid nuclei has been established within a group of three congeneric species where the total evidence almost certainly excludes polyploidy. *Liturgousa actuosa* has approximately one and one half times the amount of DNA per spermatid that is found in *L. maya* and *L. cursor;* two changes of like nature and magnitude would result in a 2 : 1 ratio with no implication of polyploidy. The possibility of a polyploid relation between low and high number karyotypes suggested by the spermatid DNA ratio between *Pseudomiopteryx* and *Stagmomantis heterogamia* is, however, open to more critical tests which it is hoped to prosecute.

DNA values as subfamily characters

Aside from the detection of polyploid relationships, the utility of DNA determinations in cytotaxonomic analysis will depend largely on whether or not the lower categories such as subfamilies possess characteristic and distinguishable relative amounts. The present data, though stemming from far too few species to answer this question, are suggestive in relation to it. The subfamilies Pseudomiopteriginae and Manteinae, as here sampled, show a wide separation between the mean values of spermatid DNA, and there is no overlapping in the range of values obtained from the two groups; should these values prove characteristic for the subfamilies, they will constitute valid cytotaxonomic criteria. In the subfamily Liturgousinae, exclusive of *Liturgousa actuosa,* the mean amount of spermatid DNA differs significantly from that of the Manteinae on one side and from the Pseudomiopteriginae on the other, although in range of values there is wide overlapping. But *Liturgousa actuosa,* in which the mean amount of DNA per spermatid does not differ significantly from that of *Stagmomantis heterogamia,* bridges the gap between Liturgousinae and Manteinae. Since taxonomical and cytological evidence utterly preclude consideration of *Liturgousa actuosa* as a bridging form between these very distantly related subfamilies, this case alone sharply restricts the potential usefulness of the relative amount of DNA in the spermatid nucleus as a cytotaxonomic criterion.

Summary

1. The relative amount of DNA per spermatid nucleus has been determined by photometric microscopic measurement in eight species of mantids, and the results are discussed in relation to their cytology and the cytotaxonomic problems they present. The mantids studied are the following: Subfamily Liturgousinae—*Litur-*

gousa maya S. & Z., *L. actuosa* Rehn, *L. cursor* Rehn; Subfamily Choeradodinae —*Choeradodis rhombicollis* Latr.; Subfamily Manteinae—*Tauromantis championi* S. & Z., *Stagmomantis carolina* Johann, *S. heterogamia* S. & Z.; and Subfamily Pseudomiopteriginae—*Pseudomiopteryx infuscata* S. & Z.

2. Of the three closely similar species of *Liturgousa* tested, *L. maya* with 16 autosomes and *L. cursor* with 32 have the same content of DNA in the spermatid nucleus, confirming the cytological evidence that no polyploid relation exists between them. The greater diameter and greater total length of metaphase chromosomes in *L. maya* as compared with *L. cursor* remain unexplained.

3. *Liturgousa actuosa* has approximately one and one half times the amount of spermatid DNA as *L. maya* and *L. cursor,* in marked disagreement with the cytological evidence for an intermediate position.

4. *Tauromantis championi* and the two species of *Stagmomantis,* of the Manteinae, are uniform in DNA spermatid values, as in karyotypes. For *Choeradodis* no reliable species constant in nuclear DNA could be determined, due to abnormalities in the material, but a value not exceeding that of the Manteinae is indicated; this is in harmony with the cytological evidence.

5. The ratio of DNA per spermatid nucleus in *Pseudomiopteryx infuscata* to that in *Stagmomantis heterogamia* is 0.47; the ratio in total length of chromosomes at the preceding metaphase is 0.39. The implications of these findings for the hypothesis that polyploidy has been involved in the evolution of high and low number karyotypes among mantids are considered.

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THE UPTAKE OF RADIOACTIVE IODINE BY ASCOPHYLLUM

SALLY KELLY [1] AND NORMAN A. BAILY [2]

Department of Plant Science, Vassar College, Poughkeepsie, N. Y., and Columbia University Radiological Research Laboratory, New York City

During the course of experiments designed to determine the relation between respiration and iodine uptake in brown algae (to be published elsewhere), it was observed that the uptake of radioactive iodine followed over a period of 15–20 hours gave a definite pattern of behavior. This has led us to interpret the uptake of iodine from sea water as involving a constant exchange process between iodine within the algae and iodine in the surrounding medium.

The interpretation of the uptake of substances by plants as a continuous exchange phenomenon by means of tracer experiments heretofore has been made by studying the movement of ions between roots and soil colloids (Jenny and Overstreet, 1938; Jenny, Overstreet and Ayers, 1939); also by movement of ions between roots and culture solution (Overstreet and Broyer, 1940; Broyer and Overstreet, 1940; Overstreet and Jacobsen, 1946; Jacobsen and Overstreet, 1947; Broyer, 1950; Russell and Martin, 1950).

The observations reported here are unique in that they involve the uptake process of a trace, rather than a major element, and they shed light on the nature of anion, rather than cation, uptake. They are also basic to the correct interpretation of further uptake experiments with the isotope.

Segments of the brown alga, *Ascophyllum nodosum* (Linn.) LeJolis, were measured for their uptake of iodine^{131} after various intervals of exposure to the isotope. Segments cut one cm. in length and washed 12–16 hours in running sea water were placed in 25 ml. volume Erlenmeyer flasks containing 3 ml. of Van't Hoff artificial sea water, to which had been added potassium iodide to give a concentration of 0.05 p.p.m. iodide (the approximate concentration in natural sea water). The isotope, iodine^{131} with no carrier added, supplied in $NaHSO_3$, was added to the solution in amounts giving approximately 14 μc. per experimental flask.

The absorbed iodine^{131} was determined after exposure to this environment for a given time interval by obtaining counts of the radioactivity present in the alga segments. Before each count was taken the segments were removed and washed by six successive rinses with a non-radioactive solution, which otherwise was similar to that in the flask. Each rinse contained 15–20 ml. of the solution. It was not possible to estimate how much of the radioactive iodine found in the rinsings came from the surface and how much from that actually absorbed by the alga. That the latter was small or at least constant was indicated by the fact that the sixth rinse showed no activity, independent of the concentration of radioactive iodine. The excess wash solution was blotted from the segments with a paper towel. The segments were then transferred to a counting pan, 10 to a pan, in such a manner that

[1] Lalor Fellow 1950, Marine Biological Laboratory, Woods Hole, Mass.

[2] This document is based on work performed under Contract AT-30-1-Gen-70 for the Atomic Energy Commission.

constant geometry was maintained for all sets of samples. After counting, they were returned to the radioactive solutions for the next time interval.

Counting rates for the solution and that remaining after the segments had been removed were determined by the use of 2 ml. aliquots in small sample pans. This constituted a thick source for the iodine betas. These counting rates made it possible to compare the shape of the curve for iodine^{131} remaining in the solution with that for iodine taken up by the alga segments.

All counting rates were determined by taking at least 2×10^3 total counts, so that the statistical error is below two per cent. The counting rates thus determined were then corrected for background and decay of the iodine^{131}.

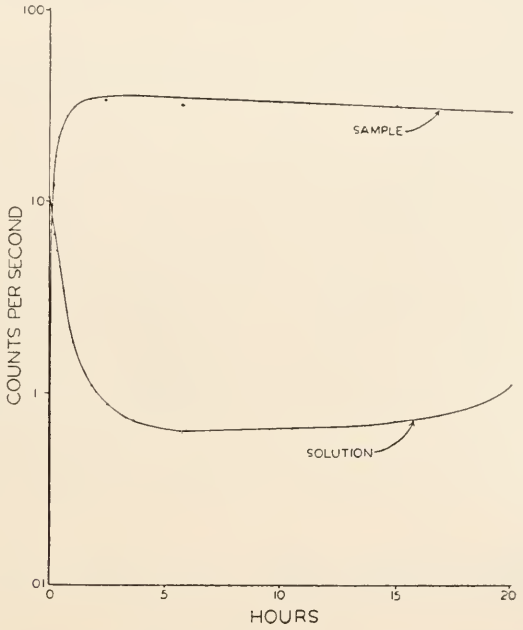

FIGURE 1. Uptake of iodine ^{131} by alga segments. This curve represents a typical experiment. Comparison of counting rates of the sample and solution is not possible because of their differences in counting geometry.

These experiments were also performed using natural sea water. This was done for the purpose of determining whether a possible difference in iodine concentration (between artificial and natural) in the sea water would affect the shape of the curves. The character of the curves did not change.

A study of Figure 1 gives the following information about the uptake of iodine^{131} by the alga segments:

1. The concentration of iodine within the segments remains the same throughout the experimental period. This is shown by the fact that a steady state of iodine^{131} content is reached eventually, and no further increase in radioactive iodine uptake is observed.

2. It is characterized by an exchange phenomenon between iodine within the

segments and iodine in the surrounding medium. This interpretation is based on the assumption that since the alga had been living for a long period of time in an iodine-present medium, natural sea water, additional uptake of iodine could occur for either of two reasons: 1) iodine is being used at a constant rate for some life process, or 2) iodine is constantly moving in and out in a dynamic exchange process. The attaining of an equilibrium, expressed in the plateau of the curve, leads us to conclude that iodine is being taken up for the latter reason.

The large slope which is obtained initially shows that this exchange is carried on at a rapid rate. The decrease in slope and the leveling off are due to the fact that initially the segments do not contain any radioactive iodine, but as the process goes on the concentration of I^{131} to I^{128} becomes equal inside the segments and in the solution. Initially, the amount of iodine^{131} within the segments is nil and the iodine being exchanged to the outside is all non-radioactive. It is replaced by a solution containing a fixed ratio of I^{131} to I^{128}. As the concentration of iodine^{131} within the segments increases, both non-radioactive and iodine^{131} are exchanged to the solution. Eventually the ratio of I^{131} to I^{128} within the segments approaches that of the surrounding medium (*i.e.*, a steady state exists) and no increase in the amount of radioactivity in the segments occurs. The slight but steady drop in radioactivity of the segments during the latter part of the experimental period has not been accounted for with any degree of certainty. In many cases iodine has been found to be unstable and to come off in gaseous form. Preliminary experiments indicate that the drop observed here is probably due to a loss of I^{131} as a gas. Curve B, showing the decrease in radioactivity of the solution corroborates the interpretation of Curve A: the initial decrease is rapid; a steady state in the solution is reached at the same time as in the segments. The rise in concentration of radioactive iodine in the solution after 16 hours may be accounted for by evaporation. Although it causes no change in the total radioactive iodine content, evaporation of the solution will increase the radioactivity per unit volume, which Curve B measures.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. I. THE CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENTS OF NORMAL TOADFISH (OPSANUS TAU)

ARNOLD LAZAROW AND SHERWIN J. COOPERSTEIN

Department of Anatomy, Western Reserve University School of Medicine, Cleveland 6, Ohio, and Marine Biological Laboratory, Woods Hole, Mass.

A determination of the metabolic characteristics of the insulin-producing beta cells of the pancreas has been undertaken in the hope that these studies may offer an explanation as to why the beta cells are selectively killed by toxic agents such as alloxan (Dunn, Sheehan and McLetchie, 1943). Several hypotheses have been advanced to explain this selectivity (Lazarow, 1949; Lazarow, Liambeis and Jan Tausch, 1950) but these must be verified by direct chemical and enzymatic analyses of the insulin-producing cells. Unfortunately, it is difficult to study the metabolism of the beta cells in mammalian species because the islet tissue is inextricably intermingled with the pancreatic acinar tissue. In teleost fish, however, the insulin-secreting cells are segregated into one or more discrete bodies called the principal islet (located in the mesentery), whereas the pancreatic acinar tissue is diffusely dispersed throughout the mesentery, along the bile ducts, and within the liver (Diamare, 1899; Rennie, 1905). The isolation of insulin from the principal islet tissue of teleost fish and its absence from the diffuse acinar tissue suggested that islet tissue was the source of insulin in mammals (McLeod, 1922). Although the fish islet tissue contains several cell types (Jackson, 1922), it may be presumed by analogy to mammalian studies that it is the beta cells which produce insulin. Since alloxan diabetes and beta cell destruction has been produced in fish (Lazarow and Berman, 1947, and also unpublished observations) it may be possible to deduce information about the metabolic specialization of the beta cells themselves by comparing normal islet tissue with islets obtained from alloxan diabetic fish (*i.e.,* islets with decreased numbers of insulin producing cells).

Unfortunately the islet tissue of a 200 gm. toadfish weighs between 1 and 2 mg. and therefore it has been necessary to undertake this study by ultramicro analytical techniques. We have begun a systematic investigation of the enzymes and metabolic activities of normal fish islet tissue; the cytochrome oxidase and succinic dehydrogenase contents are herein reported. It is hoped that these studies may help to explain the mechanism by which alloxan produces selective necrosis of the beta cells, and that perhaps they may contribute to our understanding of the etiology of human diabetes. They may also furnish information useful for the cultivation of islet tissue *in vitro,* a problem which is becoming increasingly important in view of the approaching shortage of insulin.

Methods and Materials

Mature toadfish weighing between 200 and 600 gm. were kept in a running sea water tank for one to seven days prior to use. The animals were decapitated and

samples of the various organs were removed and placed in a beaker at 0° C. The principal islet was usually found near the spleen between the layers of the mesentery. The capsule of the islet was dissected and removed. The entire islet and pieces of the other organs weighing between two and 20 mg. were placed between two layers of stretched Parafilm and weighed on a Roller-Smith Micro Torsion Balance to the nearest 1/100th of a milligram.[1]

The weighed tissue was transferred to a conical-tipped micro homogenizer (Lazarow and Portis, 1951) previously cooled to 0° C. The Parafilm was reweighed, and five volumes of fluid were added to the homogenizer with a syringe buret (Lazarow, 1950). The tissues were homogenized by hand, and after appropriate dilution the enzyme contents were determined. The order in which the different tissues were assayed was varied systematically from day to day.

Succinic dehydrogenase was determined by measuring the rate of reduction of cytochrome *c* under standardized conditions in the presence of sufficient cyanide to inhibit any cytochrome oxidase present (Cooperstein, Lazarow and Kurfess, 1950). The tissue was homogenized in 5 volumes of sodium succinate (0.5 *M* in 0.2 *M* PO_4 pH 7.4) and the homogenate was diluted with the same solution to give a final tissue dilution which ranged from 1 : 20 for islet to 1 : 300 for heart. Two determinations were carried out on each sample by mixing 10 or 20 μl. of the tissue homogenate, 10 μl. of a cyanide solution and 280 μl. of a cytochrome *c*-salt solution. The extinction was followed for three minutes at 550 mμ. The enzyme activity of a standard tissue dilution was calculated as previously described (Cooperstein, Lazarow and Kurfess, 1950) and is proportional to the Δlog [ferri-cytochrome *c*]/minute. It represents the change observed in the logarithm of the concentration of oxidized cytochrome *c* per minute if one mg. of tissue is added to 100 μl. of the cytochrome *c*-cyanide solution.[2]

Cytochrome oxidase was measured by determining the rate of oxidation of reduced cytochrome *c* (Cooperstein and Lazarow, 1951). The tissues were homogenized in 5 volumes of *M*/30 phosphate buffer, pH 7.4, and diluted with the same buffer to give a final tissue dilution ranging between 1 : 20 and 1 : 150. Two or 4 μl. of the diluted tissue preparation were added to 300 μl. of the reduced cytochrome *c* solution. The enzyme activity for a standard tissue dilution was calculated and it is proportional to the Δlog [ferro-cytochrome *c*]/minute. It represents the change in the logarithm of the concentration of reduced cytochrome *c*/minute when one mg. of tissue is suspended in 100 μl. of cytochrome *c* solution.[3]

[1] Tissues included between two layers of Parafilm do not dry out (weights remain unchanged $\pm$ .02 mg. over a two-hour period).

[2] Example: When 10 μl. of a 1 : 20 islet tissue were added to 290 μl. of a cytochrome *c*-cyanide solution and the logarithms of the extinctions (corrected for the blank) at 550 mμ were plotted against time, the Δ log [cytochrome c^{+++}]/minute was equal to .088.

$$\text{The standard enzyme activity} = \frac{\Delta\log \text{ cyt. } c \times \text{dilution} \times 300}{\text{volume tissue suspension used} \times 100}$$

$$= \frac{.088 \times 20 \times 300}{10 \qquad 100} = .528.$$

[3] Calculated as in footnote 2.

Results

The average succinic dehydrogenase activity of toadfish islet and of other selected fish tissues are arranged in the order of their activity and are shown in Table I. The final tissue dilutions under the conditions of the assay are recorded, as well as the average value of 10 determinations carried out on 10 separate fish. The standard deviations [4] and the p values are also recorded. The p value recorded represents the statistical significance of each average value as compared to the tissue of next lower activity.

It was found that islet tissue is included in the group of five tissues (testis, ovary, gill and muscle) that have the lowest succinic dehydrogenase activity. The

Table I

Succinic dehydrogenase content of toadfish tissues

Tissue	No. of fish studied	Actual tissue dilution used during assay	Average standard activity Δlog [cyto. c]/min. for a 1:100 tissue dilution	Standard deviation σ	P value compared to next lower value
Heart	10	1:9000	4.12	±0.78	<.0001
Liver	10	1:3000	1.24	±0.22	<.0001
Kidney	10	1:1500	.72	±0.17	.102
Brain	10	1:1200	.62	±0.11	<.0001
Islet	10	1:600	.26	±0.05	.88
Testis	10	1:600	.25	±0.03	.44
Gill	10	1:600	.24	±0.06	.75
Muscle	10	1:600	.23	±0.05	.60
Ovary	10	1:600	.22	±0.04	

individual values in this group are not significantly different from one another. Kidney and brain are each about 2½ times as active as islet. Liver and heart are, respectively, about 5 and 20 times as active as is islet tissue.[5]

The average cytochrome oxidase activities of tissues obtained from 10 different fish are recorded in Table II. Gill and muscle have a somewhat lower cytochrome oxidase content than does islet tissue, whereas brain, ovary and testis have a somewhat higher activity than islet (26 per cent, 23 per cent, and 44 per cent, respectively). Kidney and liver have approximately three times and heart approximately six times as much cytochrome oxidase as does islet.[5]

Cytochrome oxidase is the only known enzyme (or enzyme complex) in animal tissues involved in the biological oxidation of cytochrome *c*. By contrast, a number of dehydrogenases, including succinic dehydrogenase, are involved in the biological reduction of cytochrome *c*. It was of interest, therefore, to calculate the ratio of the enzymes which both oxidize and reduce cytochrome *c*. The succinic dehydrogenase: cytochrome oxidase ratios are plotted in Figure 1. Although the value of this ratio is very slightly greater for islet tissue than for ovary or testis,

[4] $\sqrt{\frac{\Sigma(\text{difference})^2}{N}}$.

[5] The relative enzyme activities for different tissues are unchanged when the results are converted to a dry weight basis.

TABLE II

Cytochrome oxidase content of toadfish tissues

Tissue	No. of fish	Final tissue dilution used in assay	Average standard activity Δlog [cyto. *c*]/min. for 1:100 tissue dilution	Standard error σ	P value compared to next lower tissue
Heart	10	1:22,500	10.5	1.97	<.0001
Kidney	10	1:15,000	6.06	0.84	.76
Liver	10	1:15,000	5.96	0.72	<.0001
Testis	10	1:6,000	2.64	0.25	.028
Brain	10	1:6,000	2.31	0.39	.76
Ovary	10	1:6,000	2.27	0.25	<.0001
Islet	10	1:4,500	1.83	0.21	<.0001
Gill	10	1:3,000	1.40	0.21	.0010
Muscle	10	1:3,000	1.12	0.15	

the ratio of succinic dehydrogenase (which reduces cytochrome *c*) to cytochrome oxidase (which oxidizes cytochrome *c*) is less in islet tissue than in any of the other tissues studied with the exception of kidney. Even though the succinic dehydrogenase: cytochrome oxidase ratio in toadfish kidney is somewhat lower than in islet tissue, the absolute amount of succinic dehydrogenase present was almost three times that of the islet tissue.

In order to determine whether the enzyme studies carried out on the islet tissue of fish may be extrapolated to mammalian islet tissues where direct enzyme study is difficult, we have compared the enzyme contents of corresponding tissues of the

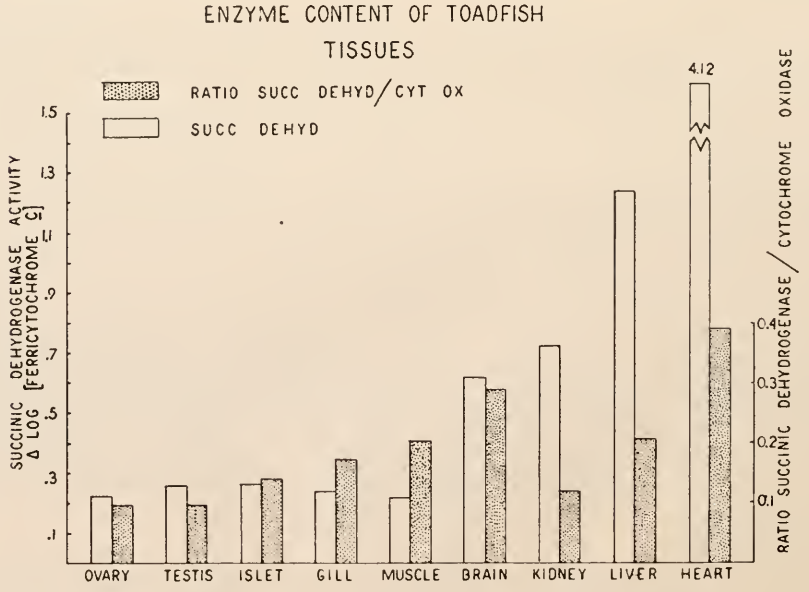

FIGURE 1.

rat and the toadfish. The enzyme activity of the most active tissue in each species (heart) is arbitrarily set at 100 per cent and the activity of the other tissues is expressed in terms of their relative activity (Table III).

Although the absolute enzyme activity of rat heart is about three times as great as toadfish heart, the relative enzyme concentrations in the corresponding tissues show good agreement in most cases. The toadfish kidney is an exception in that it contains only 18 per cent as much succinic dehydrogenase as toadfish heart, whereas rat kidney is 58 per cent as active as rat heart.

Discussion

Dunn, Sheehan and McLetchie showed in 1943 that alloxan selectively killed the beta cells in the islets of Langerhans of the pancreas. Although the exact mechanism by which alloxan destroys the pancreatic beta cells has remained obscure, it is apparent that alloxan is a selective rather than a specific poison, since larger doses

Table III

Relative enzyme content of tissues of toadfish and rat

	Cytochrome oxidase		Succinic dehydrogenase	
	Rat	Toadfish	Rat	Toadfish
Heart	100%	100%	100%	100%
Kidney	71	58	58	18
Liver	36	57	42	30
Brain	19	22	15	15
Muscle	12	11	6	6

of this compound will also produce necrosis of the liver and kidney cells (Dunn and McLetchie, 1943; Goldner and Gomori, 1943; Palay and Lazarow, 1946).

Several mechanisms have been suggested to explain the selectivity of alloxan for the beta cells (Lazarow, 1949). The metabolism of the beta cell differs from other cells in that it is continually synthesizing insulin. Insulin is a protein which has an unusually high content of sulfur amino acids (12 per cent cystine) (Du Vigneaud, 1927). Since all the sulfur of insulin is in the disulfide form, it might be expected that the metabolism of a cell synthesizing insulin would be geared to the oxidation of sulfhydryl groups to their disulfide form. Since sulfhydryl compounds such as glutathione and cysteine have been shown to protect rats against alloxan diabetes (Lazarow, 1946), and since cysteine is also used for the synthesis of glutathione, it has been postulated that the specialization of the beta cell for insulin synthesis may deplete the beta cell gluthathione and thereby increase the susceptibility of these cells to alloxan diabetes (Lazarow, 1949).

Alloxan is destroyed in the body by several reactions, one of which is its reduction to a non-diabetogenic derivative (Lazarow, Patterson and Levey, 1948), dialuric acid. This reduction, which can be accomplished by glutathione and by cysteine, can also be carried out by the reduced coenzyme, diphosphopyridine nucleotide (Cooperstein, Lazarow and Patterson, 1951). By contrast, dialuric acid can be re-oxidized to alloxan by cytochrome *c* (Cooperstein, Lazarow and Patterson,

1951) and by methylene blue (Lazarow and Liambeis, 1950). The potentiation of alloxan diabetes by methylene blue has been attributed to the reoxidation of any dialuric acid formed *in vivo* back to its diabetogenic derivative, *i.e.*, alloxan (Lazarow and Liambeis, 1950).

A further consequence of the specialization of the beta cells for insulin synthesis may be a decreased ability to destroy alloxan. If the metabolism of a cell is geared to the oxidization of sulfhydryl to disulfide, this cell may also be less able to reduce alloxan to dialuric acid (or it may rapidly reoxidize any dialuric acid formed back to alloxan). The finding by Bensley (1911) that islet tissue reduces Janus Green more slowly than does acinar tissue is in keeping with this hypothesis.

The enzyme studies reported here may likewise lend support to this hypothesis. Since dialuric acid is oxidized to an active diabetogenic compound, *i.e.*, alloxan, by the oxidized form of cytochrome *c*, it is apparent that the concentration of the oxidized form of this enzyme would play a role in determining the effective concentration of alloxan in the beta cell. The concentration of oxidized cytochrome *c* would in turn be determined by (a) the total concentration of cytochrome *c* present, by (b) the relative concentration of the enzymes which both oxidize and reduce cytochrome *c* and by (c) the spatial relations of these enzymes. Whereas the oxidation of cytochrome *c* is accomplished only by the cytochrome oxidase complex, the reduction of cytochrome *c* can be accomplished by a number of dehydrogenases. The succinic dehydrogenase complex is only one of the enzymes capable of reducing cytochrome *c*. It is apparent that both the absolute concentration of succinic dehydrogenase as well as the relative concentrations of succinic dehydrogenase to cytochrome oxidase in the beta cell would undoubtedly influence the fraction of cytochrome *c* that is in the oxidized state.

However, since other cytochrome-reducing systems would also play a role, it will be necessary to have a more complete picture of other enzymes in the islet tissue.

Since islet tissue was found to be low in succinic dehydrogenase and to have a low succinic dehydrogenase to cytochrome oxidase ratio (Fig. 1) (*i.e.*, it contains a relative abundance of cytochrome oxidase), it would appear that if the distribution of other cytochrome reducing enzymes parallels that of succinic dehydrogenase, islet tissue may well have a relatively high concentration of oxidized cytochrome *c* within the cell. Therefore, following the injection of a given dose of alloxan, any dialuric acid formed by the *in vivo* reduction of alloxan would be re-oxidized more readily and the effective concentration of alloxan within the beta cells would be higher. These cells might therefore be more susceptible to destruction by alloxan than would be the case with other tissues such as heart, liver, kidney, brain, and possibly muscle. On the basis of these enzyme studies, however, the gonads should be equally susceptible to alloxan. It must be remembered that on the other hand, the blood supply of an organ also determines the concentration of alloxan reaching that organ. The extremely rapid rate at which alloxan is destroyed does not permit equilibration to take place. On the basis of their blood supply, very vascular organs such as the islet tissue, liver, and kidney, might be expected to receive a higher total dose of alloxan than would be the case with the less vascular organs such as gonad or muscle.

It is of considerable interest that the relative enzyme activity of most of the tissues examined, using the enzyme content of the heart as 100 per cent, is the same

for rat and toadfish organs. This would suggest that the comparisons between the islet and other tissues of the toadfish may also reflect the enzyme status in mammalian tissue where direct analysis of islet tissue is more difficult. Toadfish kidney, however, differs from the other tissues studied in that it contains comparatively less succinic dehydrogenase than does rat kidney. The toadfish kidney also differs morphologically from rat kidney in that the former is aglomerular and it contains a large amount of lymphoid tissue. Whether this striking difference in enzyme content is a reflection of the differences in secretory mechanisms of glomerular and aglomerular kidneys, the presence of lymphoid tissues, the marine habitat, or the presence of gills which may take over a part of the renal excretory function, awaits further investigation.

Determinations of these enzymes in islet tissue with diminished numbers of beta cells (alloxan diabetic) will be carried out at a later date.

Summary

1. The succinic dehydrogenase and cytochrome oxidase contents of toadfish islet tissues have been determined by measuring spectrophotometrically the rate of reduction and oxidation of cytochrome *c* under standardized conditions.

2. The succinic dehydrogenase content of islet tissue was much lower than heart, liver, kidney or brain, and of the same order of activity as testis, ovary, muscle and gill.

3. The ratio of succinic dehydrogenase (a cytochrome *c*-reducing enzyme) to cytochrome oxidase (the cytochrome *c*-oxidizing enzyme) in islet tissues was low when compared to muscle, brain, liver and heart.

4. The relation of these findings to the selective destruction of the pancreatic beta cells by alloxan has been discussed.

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FURTHER STUDIES ON THE ANAEROBIC METABOLISM OF SOME FRESH WATER SNAILS [1]

BENJAMIN MEHLMAN AND THEODOR VON BRAND [2]

Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Maryland [3]

It has been shown (von Brand, Baernstein and Mehlman, 1950) that different species of fresh water snails show considerable differences in their resistance to anaerobic conditions. All species consumed carbohydrate anaerobically, but in contrast to what is known from mammalian tissues, lactic acid proved to be only a minor end-product of anaerobic carbohydrate breakdown in several species. It therefore appeared of interest to search for other metabolic end-products and in the present communication data are presented on the formation of volatile acids. This phase was selected because lower fatty acids have been found among the anaerobic metabolic end-products both of insects and worms (review of the literature in von Brand, 1946).

Material and Methods

The following species of snails were employed and where no further data are supplied, they were of the same derivation as stated previously (von Brand, Baernstein and Mehlman, 1950).

1. Pulmonates
   - Planorbidae: *Australorbis glabratus, Helisoma duryi, Planorbarius corneus, Biomphalaria boissyi, Biomphalaria pfeifferi*
   - Lymnaeidae: *Lymnaea palustris, Lymnaea natalensis*
   - Physidae: *Physa gyrina, Aplexa nitens*
2. Operculates
   - Thiaridae: *Melanoides tuberculatus*
   - Amnicolidae: *Bulimus tentaculatus,* used shortly after being shipped from Lake Erie, Michigan

The general plan of the experiments was to incubate the snails anaerobically for the maximal period compatible with their anaerobic tolerance, to determine the carbon dioxide production during the period of incubation, and to establish at the end of this period whether any volatile acids had been excreted into the medium or accumulated in the tissues of the snails.

[1] Taken in part from a thesis submitted by B. Mehlman to the Department of Chemistry, Graduate School, Georgetown University, in partial fulfillment of the requirements for the degree of Master of Science.

[2] The authors wish to express their appreciation for the contribution of snails to Dr. E. G. Berry, Mrs. M. O. Nolan, and Dr. L. Olivier of this Laboratory and Dr. H. van der Schalie of the University of Michigan.

[3] Laboratory of Tropical Diseases.

The experimental procedure employed in anaerobic incubation and in determining the carbon dioxide production has been described previously (von Brand, Baernstein and Mehlman, 1950) and the same temperature, 30° C., was used. At the end of the experiments 1 ml. of the medium (dechlorinated tap water), *i.e.,* one-half the amount used initially, was withdrawn from each Warburg flask and the steam volatile acids were determined according to the procedure of Bueding (1949). The snails of each flask were immediately transferred to 0.5 ml. of 0.1 *N* NaOH and homogenized; 2.5 ml. of 0.45 per cent $ZnSO_4$ were added and adjusted to a total volume of 4 ml. The material was therefore deproteinized essentially according to Hagedorn and Jensen (1923). After centrifugation one-half of the supernatant was used for determination of the steam distillable acids.

After these series were concluded, attempts were made to identify the volatile acids. For these experiments only two species of snails, *Australorbis glabratus* and *Helisoma duryi,* were employed. As much medium as possible was removed from the flasks and the contents of six flasks were combined. Similarly, the snails of six flasks were used together and in some instances, as indicated, the medium and the snails were combined. The acids recovered by steam distillation were separated on silica gel columns according to the procedure of Ramsey and Patterson (1945). Confirmation of the chromatographic evidence was sought by applying to the separated acids some of the microchemical tests described by Klein and Wenzl (1932).

Results

The rate of carbon dioxide production of the various species of snails in most instances was in fairly good agreement with the values reported previously (von Brand, Baernstein and Mehlman, 1950), but in a few cases, especially *Lymnaea natalensis* and *Biomphalaria boissyi,* greater differences were obtained. No definite explanation can be suggested; it is possible that differences in size or possibly feeding conditions in the aquaria prior to the experiments played a role.

Insofar as details of the carbon dioxide production are concerned, data on two sets each of *Physa gyrina* and *Helisoma duryi* are presented in Figure 1. They are representative for the non-resistant and resistant group, respectively. It is obvious that the carbon dioxide production proceeded at a fairly regular, though slowly declining, rate. This is a significant point to which we will return in the discussion.

It is very probable that the carbon dioxide values presented are minimal values, since it appears likely that some carbon dioxide retention took place. It is obviously impossible to study this question with complete snails; their calcareous shells make such experiments impossible. We tried to approach the problem by homogenizing snails (*Australorbis glabratus*) after removing them from their shells and comparing the amounts of carbon dioxide liberated into the gas phase of Warburg vessels by a definite amount of lactic acid added through the sidearm with those liberated by the same amount of acid from a bicarbonate solution. We did find that the snail tissues retained per gram tissue about 200 $mm.^3$ carbon dioxide. We are not convinced, however, that this figure is correct; snail homogenates show a pH of about 8.3. It is by no means certain that the same pH would prevail in the tissues of intact snails, since it is possible that during the homogenizing process a loss of carbon dioxide occurs. There seems, at present, no practical way of eliminating this obvi-

ous source of error. In view of this situation we do not present this phase of our experiments in detail. It may be mentioned that the tissues of Australorbis have a considerable alkaline reserve; one gram of tissue liberated upon acidification to pH 3.9 about 2270 mm.^{3} of carbon dioxide.

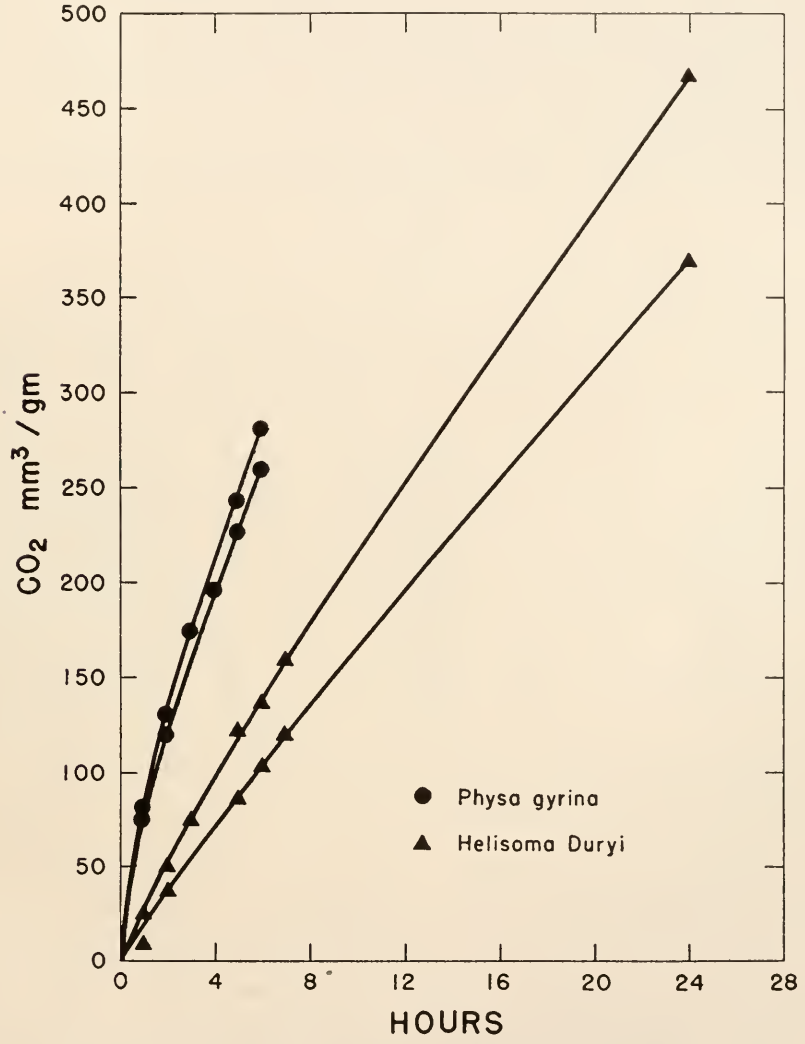

FIGURE 1. Carbon dioxide production of *Physa gyrina* and *Helisoma duryi* in relation to the length of the anaerobic period.

It was established that the snails did not have volatile acids in their tissues before they were subjected to anaerobic conditions. Control determinations with snails taken directly from the aquaria were done with each species and yielded completely negative results. After being exposed to anoxic conditions, on the contrary, significant amounts of volatile acids were found in every case both in the medium and

within the tissues (Table I). It should be noted that the species with little anaerobic resistance (*Aplexa nitens, Physa gyrina, Lymnaea palustris* and *Lymnaea natalensis*) retained a somewhat larger percentage of the acids in their tissues than the resistant ones. In most of the latter surprisingly close agreement was found, the retained acids varying only between 32 and 39 per cent of the total acids produced. *Melanoides tuberculatus* retained only 22 per cent. This snail is the species best adapted to anaerobic survival of all the species used in the present investigation, as the data on anaerobic survival presented by von Brand, Baernstein and Mehlman (1950) indicate.

The volatile acids are unquestionably a major source of the manometrically determined carbon dioxide. In the non-resistant species they are responsible for the evolution of 21 to 40 per cent of the total carbon dioxide, while the corresponding

TABLE I

CO_2 and volatile acid production of fresh water snails under anaerobic conditions

Species	No. of exper.	Anaerobiosis hours	Total CO_2 mm.³/gm.	Volatile acid in tissue % total acid	Volatile acid in medium % total acid	Total volatile acid in ml. of .005 *N*/gm.	% total CO_2 liberated from inorganic source by volatile acid
Aplexa nitens	18	6	381±23	48	52	1.39±0.12	40±2
Physa gyrina	12	6	272±10	46	54	0.78±0.06	32±2.4
Lymnaea palustris	21	6	262±20	56	44	0.50±0.05	21±1.3
Lymnaea natalensis	15	6	400±17	44	56	0.93±0.09	26±2.1
Biomphalaria boissyi	24	16	772±27	36	64	3.76±0.15	55±1.9
Biomphalaria pfeifferi	22	16	402±25	34	66	1.94±0.10	55±1.6
*Australorbis glabratus**	23;30	16	633±29	39	61	3.99±0.19	69±3.3
Helisoma duryi	15	24	436±23	38	62	2.88±0.19	72±2.9
*Helisoma duryi***	17	24	547±24	33	67	4.01±0.18	82±3.1
Planorbarius corneus	13	24	601±50	34	66	3.20±0.34	58±2.8
Melanoides tuberculatus	16	24	140± 8	22	78	0.46±0.06	35±2.8
Bulimus tentaculatus	16	24	255±24	32	68	2.07±0.18	90±3.2

* 23 experiments for the acid production, 30 experiments for the CO_2 production.

** Penicillin and streptomycin in medium.

The figures behind the plus and minus signs represent the standard error of the mean.

range is 55 to 90 in the resistant species. The only exception in this group is again *Melanoides tuberculatus,* with a value of 35 per cent.

The chromatographic separation of the acids showed that two acids were produced both by *Australorbis glabratus* and *Helisoma duryi,* only two distinct bands forming on the silica gel columns. This was established in three separate experiments with the former species in which the medium and the snails were used separately and one experiment in which medium and snails were combined. In the case of *Helisoma duryi,* lack of material prevented a separate study of medium and tissues; two combined samples were investigated. In every instance, the threshold values of the acids corresponded closely to those of acetic and propionic acids and titration of the fractions gave for both species an approximate ratio of 2:1 for the acetic and propionic acid portions, respectively. This was true in the case of Australorbis, both for the medium and the tissues.

The propionic fraction recovered from Australorbis gave with mercurous nitrate

typical crystals of the mercury salt microscopically indistinguishable from those prepared from pure propionic acid. In the case of Helisoma, the copper salt was prepared; the amounts were small, but deep blue crystals characteristic of the copper salts of lower fatty acids were obtained and they were soluble in alcohol. It seems probable, then, that we were dealing in both cases with propionic acid.

The acetic acid fractions gave with mercurous nitrate only non-characteristic crystals. This happens frequently when the slightest traces of impurities are present (Klein and Wenzl, 1932). With copper sulfate deep blue crystals were also obtained and they showed the typical insolubility in alcohol. There can hardly be a doubt, therefore, that we were dealing with acetic acid.

Discussion

The present investigation has shown that snails kept under anaerobic conditions produce volatile acids, which they partly excrete into the medium and partly retain in their tissues, the acids involved being acetic and propionic acids. The formation of the former of these acids is not too surprising since acetic acid is a rather frequent end-product of anaerobic carbohydrate utilization both in bacteria and some higher organisms. Propionic acid, on the other hand, has been found only in the case of Ascaris insofar as multicellular organisms are concerned. Since our snails were not bacteriologically sterile, the question immediately arises whether the fatty acids were actually produced by the snail tissues or were due to bacterial activity which conceivably might have transformed lactic acid derived from the anaerobic metabolism of the snails into the lower fatty acids. It is well known that this question gave rise to a prolonged controversy in the case of parasitic worms. It has recently been decided unequivocally by Epps, Weiner and Bueding (1950), who succeeded in sterilizing *Ascaris lumbricoides* by means of antibiotics and found that these worms did produce lower fatty acids, among them acetic and propionic acid.

We carried out a series of experiments with *Helisoma duryi* kept in dechlorinated tap water containing 2000 units of penicillin and 10,000 units of streptomycin. As the data of Table I show, these snails produced somewhat more carbon dioxide and volatile acid than the nontreated snails, but even this medium yielded upon transfer to a nutritive medium and subsequent incubation some bacterial colonies, and there is no justification to assume that the alimentary tract of the snails would have been sterilized. We abandoned further attempts to sterilize snails because we gained the impression that prolonged stay in the antibiotic solutions, though not killing the snails, affected them adversely.

The Australorbis used in the chromatography experiments were laboratory reared, while the Helisoma were freshly collected from large tanks in Kenilworth Gardens, Md. Both nevertheless yielded the same acids; if bacterial activity was involved, both species would presumably have had to carry the same bacterial flora. Both species also accumulate acids in their body. The internal tissues of snails are presumably sterile; one would then have to assume that the metabolic end-products would have been transported first to contaminated organs, the alimentary tract and perhaps the outside tissues, and have been transformed there to fatty acids. These assumptions, although not impossible, are none too probable. It should also be remembered that the carbon dioxide production of the anaerobic snails declined somewhat with time; if a bacterial population would have been built up, one would have

expected rather an increase in rate. We are inclined to assume tentatively that the acids originated from the metabolism of the snails, but we concede that the final decision will have to wait for work on bacteriologically sterile snails.

We wish to emphasize strongly, however, that the biological implications of our findings are unaffected by the question of whether the snails themselves produced the volatile acids or not, since in nature no sterile snails can be expected to occur. We have pointed out previously (von Brand, Baernstein and Mehlman, 1950) that the non-resistant species, in contrast to the resistant ones, accumulated lactic acid within their tissues and we assumed that this difference may be one of the factors explaining the difference in resistance to anaerobiosis. We pointed out, however, that lactic acid could not be responsible alone for the death of the snails, because the resistant species also ultimately died of asphyxiation. Our present results would seem to indicate that the marked accumulation of volatile acids in the tissues may

TABLE II

Percentage of anaerobic carbon dioxide liberated from inorganic sources

Species	Per cent of total carbon dioxide liberated from inorganic sources		
	By lactic acid*	By volatile acids	Total
Aplexa nitens	67	40	107
Physa gyrina	38	32	70
Lymnaea natalensis	139	26	165
Lymnaea palustris	41	21	62
Biomphalaria boissyi	25	55	80
Biomphalaria pfeifferi	17	55	72
Australorbis glabratus	13	69	82
Helisoma duryi	4	72	76
Planorbarius corneus	3	58	61
Melanoides tuberculatus	17	35	52

* Data from von Brand, Baernstein and Mehlman (1950).

have a bearing on the problem. The lower fatty acids are probably less toxic than lactic acid, but still if they accumulate to a certain level they may be harmful. The difference in the respective amounts of end-products of various toxicities within the tissues of different species of snails may well be at the root of the differences in anaerobic resistance. Whether it is the sole factor involved cannot be stated with any degree of certainty, however.

It would obviously be premature to speculate about the mode of formation of the volatile acids, but a brief discussion of the carbon dioxide picture seems appropriate. We have pointed out in our previous paper that the carbon dioxide evolved by the non-resistant species is largely of direct inorganic origin, that is, liberated by lactic acid from bicarbonate. The relevant data concerning this point are shown again in Table II. The third column shows the amounts of carbon dioxide liberated from inorganic sources by the volatile acids. It is now clear that in all species a very large percentage of the carbon dioxide is of direct inorganic origin. It is possible or even probable that the remainder of the carbon dioxide unaccounted from this source is true respiratory carbon dioxide, carbon dioxide derived from the break-

down of carbohydrate. No accurate data along this line can be calculated, however, because of our inability to elucidate adequately the question of carbon dioxide retention by the tissues. It may be pointed out that this source of error may be responsible for the fact that less carbon dioxide was found in the cases of *Lymnaea natalensis* and *Aplexa nitens* than corresponds to the acids produced.

Summary

1. Fresh water snails exposed to anaerobic conditions produce volatile acids which are partly excreted into the medium and partly accumulate in the tissues.

2. The acids formed by *Australorbis glabratus* and *Helisoma duryi* were identified by chromatographic means and crystallographic data as propionic and acetic acids.

3. While bacterial formation of these acids cannot be excluded categorically, some evidence is adduced to the effect that they may be produced by the snail tissues.

4. The evidence indicates that the species not resistant to anaerobiosis are killed primarily by the accumulation of lactic acid, while the resistant species are more tolerant to the lack of oxygen due to the fact that they accumulate in their tissues the less toxic fatty acids rather than lactic acid.

5. Most of the carbon dioxide evolved by anaerobically kept snails is of direct inorganic origin.

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THE CHAETOGNATHA OF THE WEST COAST OF FLORIDA[1]

E. LOWE PIERCE

Department of Biology, University of Florida, Gainesville, Florida

Very little work has been undertaken on the Chaetognatha of the Gulf of Mexico. A short publication by Ritter-Zahony (1910) on specimens collected in the region of the Dry Tortugas, Florida, is the single paper available dealing directly with chaetognaths which might be considered as inhabitants of the Gulf. Conant (1895, 1896) has published notes and descriptions of species from the West Indies and Beaufort, North Carolina, representatives of which are found in the Gulf plankton. Davis (1949) and King (1949) have published preliminary reports on the plankton along the coast of Florida which include brief mention of the chaetognaths. Certain species of chaetognaths found in the Gulf have a cosmopolitan distribution. Reference to these species collected in other regions may be found in many papers.

The purpose of this study was to determine the species present along the west coast of Florida, their distribution, abundance and breeding seasons through an annual cycle. From previous sampling it was known that an appreciable gradient in salinity extended from the coastline to 15 miles or more offshore. It was desirable, therefore, to set up stations within this zone of variable salinity and to determine its effects on chaetognath distribution. Moreover, by attempting to locate the stations in the same relative salinity a truer comparison could be made between the populations of chaetognaths at different points along the coast.

Beginning in November, 1948 and continuing through January, 1950 plankton collections were made at monthly intervals from three well-spaced areas along the west coast. Ft. Myers Beach, Bradenton Beach and Cedar Key were selected as base points and from each of these bases three collecting stations were established approximately ½, 5 and 15 miles offshore (Fig. 1). For convenience in reference in this study, the bases are indicated by the numbers 1 (Ft. Myers Beach), 2 (Bradenton Beach) and 3 (Cedar Key), and the stations by A, B, and C from inshore to offshore, respectively. Thus, the inshore station at Ft. Myers is indicated by 1A and the offshore station at Cedar Key by 3C. These stations were all located on the inner border of the continental shelf which on the west coast of

[1] I am indebted to the University of Florida for providing funds which made this work possible. Dr. Ralph A. Morgen, Director of the Engineering and Industrial Experiment Station and Dr. C. Francis Byers, Professor of Biology, cooperated in the organization of this project. Mr. J. M. Thomson, Research Officer, Fisheries Research Station, Dunwich, Queensland, Australia, sent me identified specimens of chaetognaths taken from Australian waters. Dr. J. H. Fraser, Marine Laboratory, Aberdeen, Scotland, examined a number of specimens sent to him for identification and offered several useful suggestions. Dr. Fenner A. Chase, Jr., Curator, Division of Marine Invertebrates, United States National Museum, allowed me to work in the museum and to examine the identified chaetognaths stored there. Mr. E. D. McRae aided in the collection of the samples. I am grateful to Miss Esther Coogle, staff artist, for the careful drawings in this paper.

Part of the cost of publication was furnished by the University of Florida.

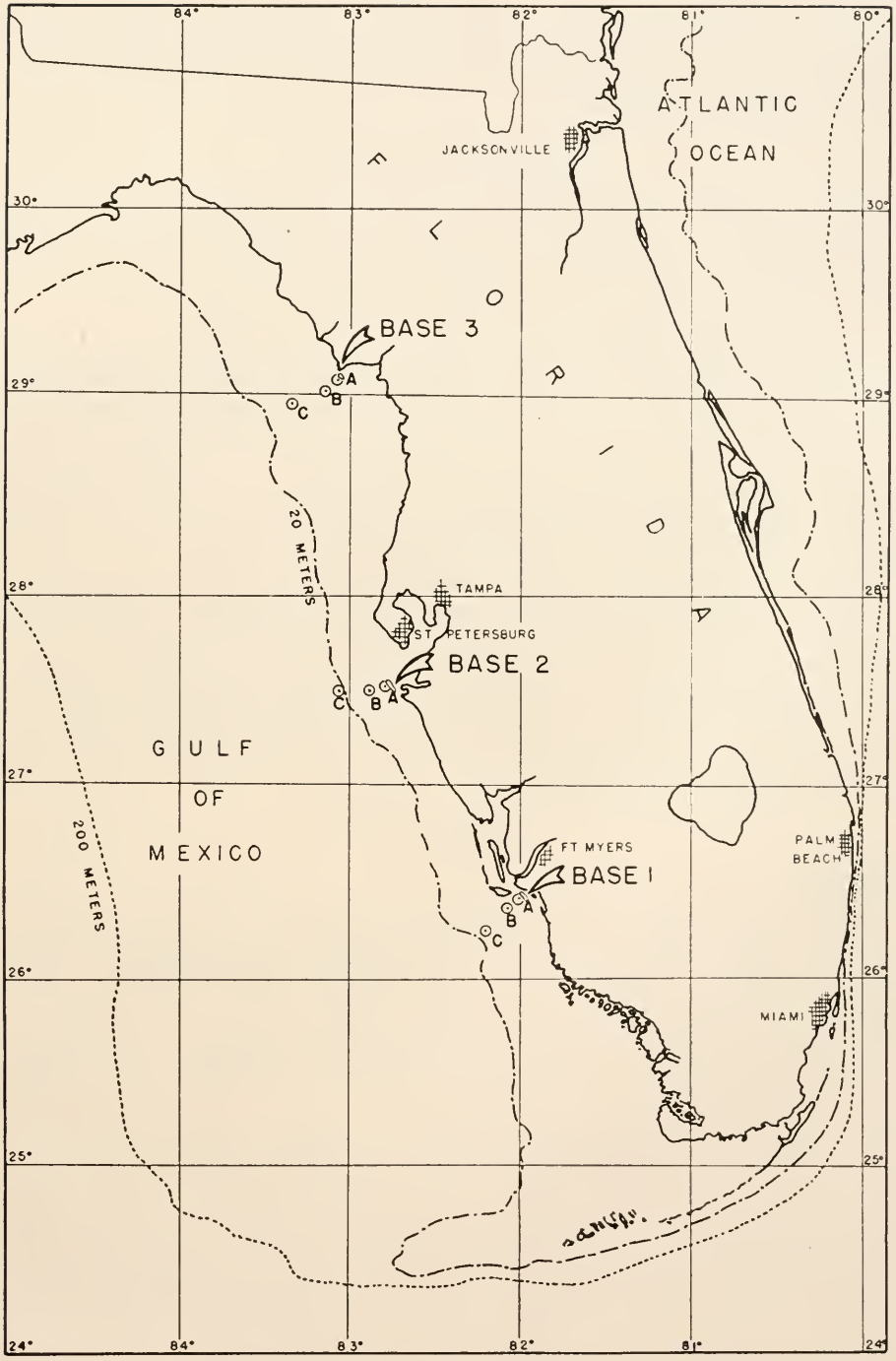

FIGURE 1. Map of Florida with the bases and stations on the west coast indicated.

Florida is a broad, gently sloping expanse of sea bottom extending 75 miles or more from the coast. The waters which were sampled in this study were all relatively shallow, ranging in depth from 4–5 meters at stations A, 6–10 meters at stations B and 12–18 meters at stations C.

Collecting Procedures

Surface temperatures and salinities were taken at each station, and at all offshore stations (C) the temperature and salinity of the bottom water were measured as well. Salinities were obtained by hydrometers of the type used by the United States Coast and Geodetic Survey, which allow the measurement of salinity to one-tenth of a part per thousand.

Two techniques were employed for collecting plankton samples. The principal method was a quantitative one which involved the use of the Clarke-Bumpus quantitative plankton sampler (Clarke and Bumpus, 1940). Early attempts to use a number 20 plankton net on this sampler were unsuccessful because of clogging of the meshes of the net, especially at the inshore stations. An oblique towing was made from surface to bottom at all A and B stations. At each C station one quantitative sample was taken from the bottom to mid-depth, and another from mid-depth to the surface to learn if there was an unequal distribution of chaetognaths between the surface and bottom layers of water.

The second type of net employed was a half-meter net made of a number 6 bolting cloth (74 meshes/inch). This was designed to catch a much larger sample than the quantitative net and would be more likely to capture specimens which were present in reduced numbers. This net was towed obliquely from surface to bottom at all stations. All nets were pulled for about ten minutes and at a speed of one knot approximately. The quantitative net customarily sampled between one and two cubic meters of water in this period.

Hydrography

Temperature

The records for surface water temperatures for stations A and C of bases 1, 2 and 3 are shown in Figure 2.[2] The greatest annual range in temperature at each base was found at the inshore stations. These ranges were as follows: station 1A, 19.8–29.7° C.; station 2A, 19.9–29° C.; station 3A, 15.9–29.6° C. The range at the offshore stations was somewhat less. The maximum water temperatures compared very closely at all stations. They varied from 29.0° C. to 29.9° C. The minimum temperatures varied from 15.9° C. at 3A to 20.8° C. at 1B. Whereas the warmest water temperatures were similar at all stations, the coldest water by several degrees was found at the Cedar Key station. The greater annual temperature range noted at this station is the result, therefore, of cooler water temperatures. The temperatures recorded at Cedar Key (3A) are comparable to the records of the United States Coast and Geodetic Survey (1944) taken in the vicinity of 3A.

The noticeably irregular lines of the graphs in winter as compared to the remainder of the year show the effect of the short periods of relatively cold weather

[2] Because of the normally small variation in temperature between the A and C stations, it was considered inadvisable to graph the figures for the B stations which were intermediate.

which normally occur in this area in the winter. The shallow inshore waters respond rather quickly to chilling by the cold air.

The temperature of the surface water and the water about one meter from the bottom was measured at all the C stations. Bottom temperatures in most cases agreed within a few tenths of a degree with surface temperatures and were usually cooler by a few tenths of a degree. In a number of instances, however, the surface water was the cooler of the two or the water was the same temperature from surface to bottom. These observations, as well as the information received from salinity records and plankton counts, indicate that the water from shore, at least as far as

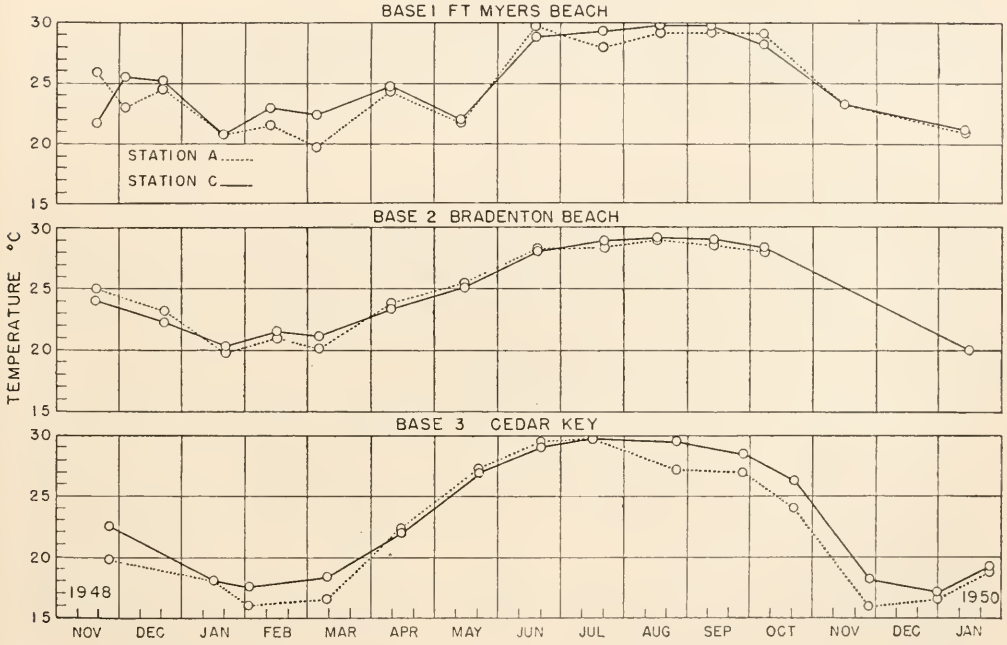

FIGURE 2. Monthly surface water temperatures at all A and C stations. Temperatures at the B stations were intermediate and were omitted to prevent confusion.

the C stations, is almost continuously undergoing mixing and that little stratification is allowed to take place at any season of the year.

Salinity

An inspection of Figure 3 shows that the salinity of the inshore waters is notably more reduced than that of the offshore stations because the run-off from the land causes appreciable dilution of the shallow coastal water. Bar graphs of rainfall have been included for Ft. Myers Beach and Cedar Key to show the direct effects of rainfall on salinity at stations 1A and 3A.[3] The range in salinity is much greater at the inshore stations. This is less noticeable at station 2A because it was rela-

[3] U. S. Dept. of Commerce, Weather Bureau. 1948–50. Climatological Data. Florida. Vol. 52, No. 13, Vol. 53 and Vol. 54.

tively farther away from the mainland drainage than either 1A or 3A. The lowest salinities were usually at 3A and were in part the result of the outflow of the neighboring Suwannee and Waccasassa Rivers in addition to the run-off from many smaller streams and marshes in that area. The highest salinities were found at stations 1C and 2C and compare closely with salinities encountered on one collecting trip which extended about 40 miles offshore in the Bradenton Beach area. The effects of dilution on salinity were noticeable at 3C.

The effects of the tide on salinity were measured at station 3A on three occasions between successive high and low tides. The salinity range in these cases was

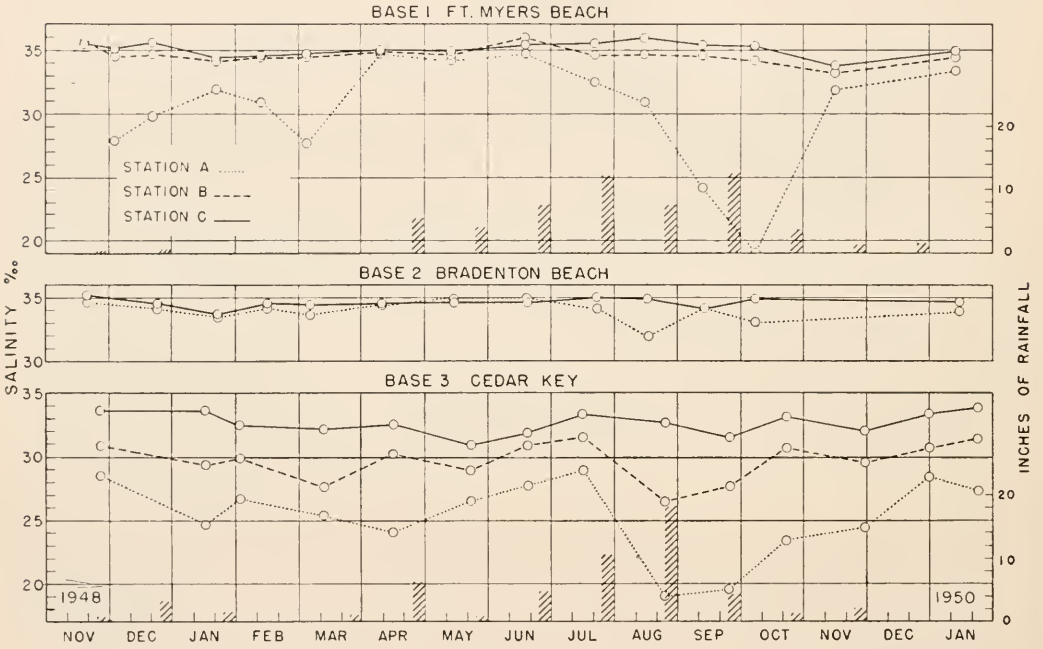

FIGURE 3. Monthly surface salinities. The bar graphs indicate total monthly rainfall at Ft. Myers and Cedar Key.

1.5‰, 1.9‰, 3.5‰. At all inshore stations these variations with the tide are undoubtedly responsible for some irregularities in the curves of salinity; however, they do not account for the more pronounced trends which are dependent to a large extent on rainfall.

Measurements and Maturity Stages

Measuring and staining

All chaetognaths were measured to the nearest millimeter. Measurements of length were made exclusive of the caudal fin. In order to see in detail certain features of the anatomy, especially the corona and gonads, it is frenquently necessary to stain the specimens. A convenient stain for this purpose is a one per cent solution of methylene blue in which the specimen need be immersed only a few

minutes (Thomson, 1947). A more permanent stain which is effective for the ovaries and testes is alum carmine. The gonads are readily visible in specimens which have been left in a weak solution of this stain for several hours or overnight. If the tissues become too deeply stained, they may be de-stained rapidly by immersing the specimen in a few milliliters of water to which has been added a drop of hydrochloric acid.

It was found that by placing a chaetognath in a 2 or 3 per cent solution of iodine and potassium iodide for a few minutes, its teeth and hooks, ordinarily stain resistant, became slightly stained. This is an aid in counting the teeth when the chaetognath is held against a white background. The iodine, moreover, has the effect of giving the flesh a putty-like consistency which allows the teeth to be pressed into a more visible position for counting. The use of the above technique, coupled with observation of the specimens under a wide-field dissecting microscope capable of 100 × magnification, normally allows specimens as small as 5 mm. to be examined with success.

Maturity stages

Several criteria have been suggested by a number of investigators for separating the hermaphroditic chaetognaths into stages which represent progressive states of maturity. Kramp (1917) initially proposed recognition of four stages of maturity. Russell (1932) reduced Kramp's stages to three as a result of studies on *Sagitta elegans*. Wimpenny (1936) has defined four stages of maturity which are rather similar to Russell's. Thomson (1947) proposed a subdivision of maturity into four groups based only on development of the ovaries. While there is a considerable basic similarity in the various systems proposed, attention must be paid to the description of each stage specified by an investigator because it is not necessarily identical with similarly numbered stages of other workers.

In this paper three stages of maturity are recognized. These are as similar to Russell's stages as the individual variations found in the developing gonads of different species will allow. Three stages are sufficient to give a reliable picture of breeding and the inclusion of more stages adds to the work of separating the chaetognaths without commensurate returns in information.

In this problem either the entire sample or a fraction, usually containing 30–40 chaetognaths, was taken from the collection made at each station. All the specimens selected were measured, stained and graded into maturity stages. Because a very appreciable difference may be observed in the state of maturity for any given body length, it is believed that more valid conclusions may be reached by this procedure than by comparing the length and stage of maturity from a limited number of specimens and assuming that the remainder of the sample can be divided into maturity stages on the basis of length measurements alone.

The stages of maturity recognized in this study are:

Stage I. Immature chaetognaths which show no clusters of maturing sperm cells or spermatozoa in the tail cavity. Ovaries small or indistinct.

Stage II. Clusters of sperm cells in tail cavity. Ovaries show signs of growth. No enlarged eggs present at this stage.

Stage III. Ovaries have increased in length and some of the individual eggs have increased appreciably in size.

There is a considerable difference in the shape and size of the ovaries and in the size of the mature ova. In the case of two of the species collected, *Sagitta hispida* and *S. helenae,* the lengthening of the ovary was pronounced in the mature forms, whereas the increase in size of the individual eggs was gradual, the eggs never

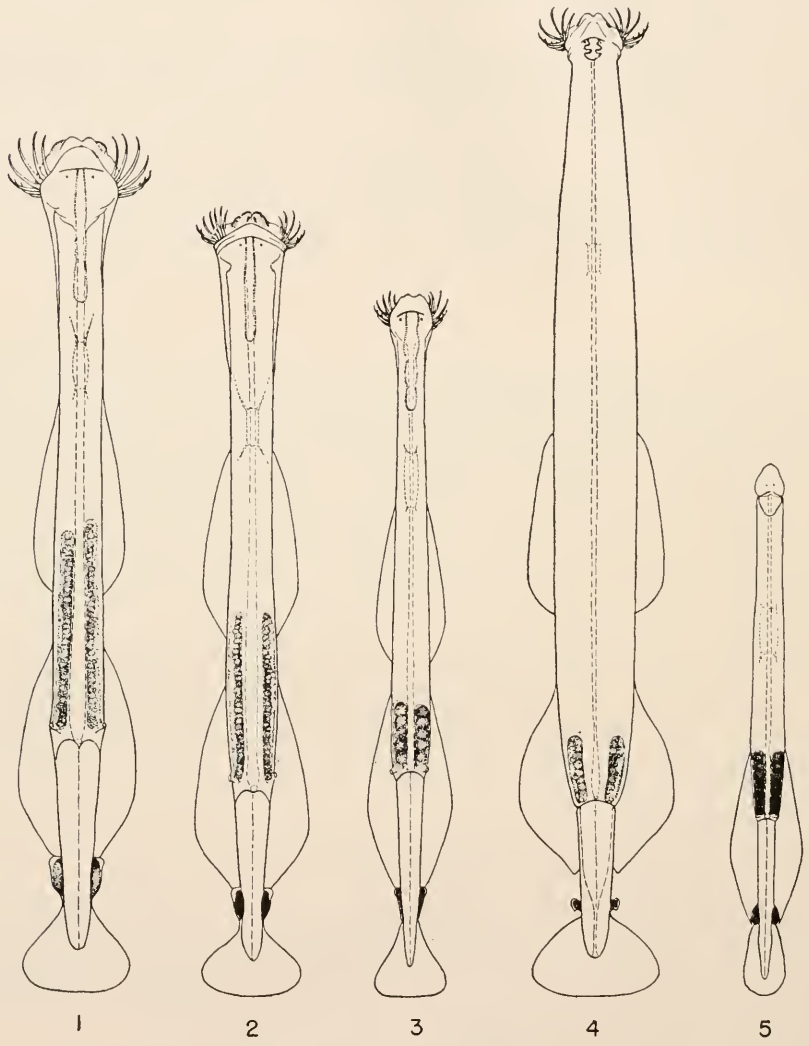

FIGURE 4. The species of chaetognaths collected along the west coast of Florida. 1. *S. hispida* 12×; 2. *S. helenae* 6×; 3. *S. tenuis* 12×; 4. *S. enflata* 6×; 5. *K. pacifica* 12×.

becoming very large. It was therefore decided to define the beginning of Stage III for these species as that point at which the lengthening ovary reached as far forward as the posterior end of the anterior fin. In the remaining species collected the ovaries never extended very far forward in the body cavity, but the size of the

developing eggs was more evident and this was the criterion used to define Stage III as indicated above.

The differences in the details of the development of the gonads of the various species of chaetognaths are very noticeable. Not only are there differences in rate of development and size of the ovaries, but in the case of *S. enflata,* for example, the sperm balls which normally circulate freely in the tail cavity become concentrated in two packed masses at the extremity of the tail segment.

West Coast Chaetognaths

List of species. Two genera and five species of chaetognaths were collected from the west coast of Florida during the course of this investigation. These were: *Sagitta hispida* Conant, 1895; *Sagitta helenae* Ritter-Zahony, 1910; *Sagitta tenuis* Conant, 1896; *Sagitta enflata* Grassi, 1881; *Krohnitta pacifica* (Aida), 1897.

Sagitta hispida Conant (Fig. 4)

Sagitta hispida Conant, 1895, p. 290, 1896, p. 214; Fowler, 1906, p. 58; Michael, 1911, p. 27, 1919, p. 239; Burfield and Harvey, 1926, p. 96.

Sagitta robusta Ritter-Zahony, 1910, p. 136.

Description. S. hispida was originally but incompletely described by Conant (1895) from specimens obtained at Beaufort, North Carolina. This species has

Table 1

Measurements of S. hispida from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
4–5	26–27	6–8	4–6	10–11
6–7	27–28	7–9	5–6	7–10
8–9	26–29	7–8	6–9	10–12
10–11	26–29	7–8	6–9	10–12
12–13	26	8	7	10

been mis-identified on more than one occasion in the literature. Aida (1897) reported it from the harbor of Misaki in Japan. Tokioka (1939), who has studied the chaetognath fauna of Japan extensively, synonymizes *S. hispida* Aida with *S. robusta* Doncaster. Burfield and Harvey (1926) reported *S. hispida* from the Indian Ocean but their statement that the posterior fins do not extend to the seminal vesicles and that there is slightly more fin on the trunk than on the tail is not in agreement with Conant's figure or my data.

There are three specimens of *S. hispida* in the United States National Museum, which were deposited there in 1896 by F. S. Conant (Cat. No. 4984).[4] The dental formulae could not be determined without damaging these specimens, all of which were not in good condition when examined; however, the outline of the body, the position and shape of the fins and the seminal vesicles could be seen clearly on one, and these features agreed with Conant's (1895) figure and description for this

[4] Through the courtesy of Dr. Fenner A. Chase, Jr., Curator, Division of Marine Invertebrates, I was allowed to examine these specimens.

TABLE II

Measurements of S. hispida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
Three specimens from Beaufort Inlet, N. C.[5]				
5	29	7	4	8
7	29	6	6	9
8	27	6	6	9
Data from Conant, 1895, Beaufort, N. C.				
7–11	33	8–9	4–5	8–15

TABLE III

The occurrence of chaetognaths at Base 1

Figures represent numbers per cubic meter

Species at Stations A, B, C, C'*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'
1948																				
19 Nov.	111	0	–	–	0	48	–	–	40	11	–	–	7	3	–	–	3	22	–	–
22 Dec.	10	0	0	0	0	48	6	7	10	28	13	41	0	34	83	45	1	11	6	0
1949																				
21 Jan.	2	99	158	48	0	4	4	3	2	106	6	8	0	0	19	20	0	2	3	0
12 Feb.	6	49	0	0	0	1	47	44	0	19	6	19	0	1	28	19	0	2	0	2
4 Mar.	41	15	0	0	0	0	45	36	41	37	7	25	0	0	24	67	0	0	3	0
9 Apr.	78	53	0	0	0	0	53	100	7	117	10	7	0	11	13	4	0	0	0	0
13 May	197	33	0	0	0	0	96	116	0	2	24	0	0	0	28	19	0	0	5	5
18 June	23	68	0	0	0	0	325	197	0	5	8	9	0	0	17	17	0	0	0	0
21 July	52	18	0	0	0	0	32	20	0	0	0	0	0	0	0	1	0	0	0	0
17 Aug.	41	73	–	0	0	0	–	25	0	0	–	0	0	0	–	0	0	0	0	0
12 Sept.	141	77	44	56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7 Oct.	46	44	0	0	0	0	34	30	0	0	0	0	0	0	0	3	0	0	0	0
15 Nov.	130	35	0	–	0	17	31	0	4	0	0	–	0	78	6	–	0	28	0	–

* C net towed from 0 to 8 meters, C' net towed from 8 meters to bottom. – Indicates absence of sample at that station.

species. Conant's use of a small specimen with the hood covering the head gives the impression that the head is distinctly narrower than it appears when the hooks are extended and the hood drawn back. As indicated in the table of measurements, a few specimens of *S. hispida* were secured from the entrance to Beaufort Harbor, the type locality.[5] These are in essential agreement with Conant's specimens and with the material which has been collected from the west coast of Florida.

[5] Specimens supplied through the courtesy of Dr. W. H. Sutcliffe, Jr., Duke Marine Laboratory, Beaufort, N. C.

S. hispida is a smaller than average chaetognath. The size at maturity ranges between 5 and 12 mm., 8 mm. being common. The body is rigid, collarette conspicuous, corona elongate, extending from a point anterior to the eyes well down on the body, but not reaching as far as the ventral ganglion. The fins are completely supplied with rays. The posterior fin touches the seminal vesicles in mature specimens. The number of anterior teeth is variable, ranging from 4–6 in small forms to as many as 9 in the larger specimens. These teeth are held flat against the head, a feature which in addition to the smaller number allows this species to be distinguished from *S. helenae*.

TABLE IV

The occurrence of chaetognaths at Base 2

Figures represent numbers per cubic meter

Species at Stations A, B, C, C'*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'
1948																				
20 Nov.	—	18	0	—	—	11	77	0	—	6	11	—	—	0	23	—	—	0	11	—
23 Dec.	—	22	0	0	—	1	26	3	—	1	18	16	—	0	25	13	—	0	8	1
1949																				
22 Jan.	15	0	0	0	0	15	4	9	7	4	3	9	0	4	9	88	0	0	0	2
13 Feb.	57	11	0	0	0	0	15	28	4	0	12	34	0	4	91	333	0	0	0	0
5 Mar.	33	0	0	0	0	0	11	7	14	0	0	0	0	0	8	32	0	0	0	0
10 Apr.	12	27	0	0	0	10	11	8	12	14	7	26	0	0	2	11	0	12	0	0
14 May	45	15	0	0	0	5	4	14	5	9	0	1	0	0	4	10	0	0	0	0
19 June	46	7	0	0	0	18	46	75	0	0	5	13	0	0	2	8	0	0	0	0
22 July	9	24	0	0	0	0	23	0	0	6	0	0	0	0	0	0	0	0	0	0
16 Aug.	54	0	0	0	0	58	50	25	0	2	0	0	0	0	0	0	0	0	0	4
13 Sept.	112	0	0	0	0	15	45	29	4	3	3	7	0	0	0	2	0	0	3	2
7 Oct.	29	17	21	69	0	2	0	19	6	2	0	0	0	0	2	0	0	8	1	19
1950																				
Jan.	49	4	0	—	0	2	32	—	4	3	11	—	0	3	9	—	0	1	1	—

* C net towed from 0 to 8 meters, C' net towed from 8 meters to bottom. — Indicates absence of sample at that station.

Distribution. This species was taken consistently at all the A stations. A comparison of Tables I, II, III and Figure 3 shows the direct relationship of this species with the shallow, less saline coastal water. No immediate connection could be seen, however, between the month to month fluctuation of salinity at any one station and the appearance or disappearance of *S. hispida* at that station. Moreover, despite the similarities in the salinity at stations 2A and 2C, this species was almost always abundant at the inshore station (2A) and with one exception was never taken at the offshore station (2C). This suggests that some factors in addition to salinity are operating to control the distribution of chaetognaths in this area. It does not appear likely that temperature exerts any appreciable effect; for example, compare the similar temperatures at 2A and 2C (Fig. 2).

In addition to its occurrence at the regular stations, *S. hispida* was collected just off the tip of Cape Sable, August, 1949; from a point midway along the Florida Keys (Bow Channel, Sugarloaf Key) January, 1947; a channel one mile north of Key West, June, 1946; and, in company with *S. helenae*, at a point about 12 miles northwest of Key West, July, 1946. These records further indicate its preference for inshore sea water of reduced salinity.

Breeding. In the warm waters of the Gulf, *S. hispida* breeds to some extent the year round (Table IV). There was no month in which a high percentage of

TABLE V

The occurrence of chaetognaths at Base 3

Figures represent numbers per cubic meter

Species at Stations A, B, C, C'*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'
1948																				
26 Nov.	—	57	0	0	—	0	21	27	—	0	8	0	—	0	3	0	—	0	0	0
Dec.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1949																				
16 Jan.	—	—	—	0	—	—	—	80	—	—	—	0	—	—	—	88	—	—	—	0
2 Feb.	18	27	122	88	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12 Mar.	46	28	0	0	0	0	31	71	0	0	11	28	0	1	2	4	0	0	0	0
15 Apr.	52	37	0	16	0	0	10	16	4	7	24	64	0	0	0	5	0	0	0	0
22 May	223	77	0	0	0	0	53	95	0	8	1	2	0	0	3	0	0	0	0	0
19 June	481	106	14	47	0	0	14	31	0	2	20	25	0	0	0	0	0	0	0	0
15 July	53	45	15	23	0	0	0	0	0	1	15	4	0	0	2	0	0	0	0	0
24 Aug.	—	37	8	17	—	0	2	0	—	0	2	2	—	0	0	0	—	0	0	0
25 Sept.	—	160	62	24	—	0	0	0	—	0	0	0	—	0	0	0	—	0	0	0
22 Oct.	—	94	22	31	—	0	0	0	—	0	0	0	—	0	0	0	—	0	0	0
29 Nov.	21	2	—	—	0	0	—	—	3	0	—	—	0	0	—	—	0	0	—	—
31 Dec.	—	13	0	—	—	0	22	—	—	0	1	—	—	0	2	—	—	0	1	—
1950																				
Jan.	13	2	2	—	0	0	17	—	—	0	8	—	—	0	1	—	—	0	1	—

* C net towed from 0 to 8 meters, C' net towed from 8 meters to bottom. — Indicates absence of sample at that station.

Stage I individuals were not present. In March and July, 11 per cent were mature Stage III forms. There is some evidence, judging by the increase in young as well as by the appearance of mature specimens, that reproduction increased somewhat in mid-winter and again in the spring, but no well-defined breeding season could be discerned. This is in contrast to studies which have been made in northern waters, where seasonal breeding in certain species is seen more clearly (Pierce, 1941; Clarke *et al.*, 1943).

Abundance. The numbers of *S. hispida* captured per cubic meter of water sieved are shown in Tables I, II, and III. There was a sizable increase in the population in May at all A stations and in June at 2A and 3A; however, there were

TABLE VI

Stages of maturity for S. hispida
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total
	No. spec.	%	No. spec.	%	No. spec.	%	
1948							
Nov.	62	75	17	20	4	5	83
Dec.	31	72	12	28	0	0	43
1949							
Jan.	187	91	17	8	3	1	207
Feb.	168	84	29	14	3	2	200
Mar.	42	50	33	39	9	11	84
Apr.	79	54	67	45	1	1	147
May	155	80	38	19	2	1	195
June	141	49	139	48	9	3	289
July	99	54	65	35	19	11	188
Aug.	213	78	49	18	10	4	273
Sept.	370	74	116	23	16	3	502
Oct.	156	73	50	23	8	4	214
Nov.	54	83	10	15	1	2	65
Dec.	72	95	4	5	0	0	76
1950							
Jan.	33	73	12	27	0	0	45

still numbers of young Stage I forms present during the other months of the year (Table IV) which is consistent with the evidence that breeding is continuous the year round.

A comparison may be made of the abundance of *S. elegans* over Georges Bank, off Massachusetts, during five cruises made in 1940 (Clarke *et al.*, 1943) with the number of *S. hispida* taken at three stations on the west coast for corresponding months. The maximum concentration of *S. elegans* for each cruise has been selected for comparison.

TABLE VII

*Comparison of catches of S. elegans from Georges Bank, Massachusetts and S. hispida from the west coast of Florida**

	January	March	April	May	June
Georges Bank					
1940	26	5	11	43	112
West Coast of Florida					
1949					
Station 1A	2	41	78	197	23
Station 2A	15	33	12	45	46
Station 3A	13	46	52	223	481

* Figures represent individuals per cubic meter.

With some exceptions, the numbers of individuals are higher along the west coast than they are over Georges Bank. Of course, two different species are being compared; nonetheless, the data do suggest that population increases in northern waters are not always greater than population increases for similar species in the south during the breeding season.

Sagitta helenae Ritter-Zahony (Fig. 4)

Sagitta helenae Ritter-Zahony, 1910, p. 134, 1911a, pp. 18, 1911b, p. 20; Michael, 1919, p. 239; Thomson, 1947, p. 36; Moore, 1949, p. 26.

Description. This species was described by Ritter-Zahony (1910) from specimens taken near Dry Tortugas, Florida. It is a medium sized form with rather heavy body musculature and a large number of anterior teeth. The number and arrangement of these teeth allow it to be distinguished from the very similar though smaller *S. hispida* Conant. The anterior teeth in *S. helenae* are more abundant for any given size than are the teeth in *S. hispida.* In the 6–10 mm. range the difference is apt to be two to three anterior teeth; in the larger specimens

TABLE VIII

Measurements of S. helenae from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
6–7	25–27	6–7	8–10	7–10
8–9	26–28	6–8	9–12	9–13
10–11	25–26	7–8	11–16	11–12
12–13	24	7	15	11–12
14–15	24–25	7–8	15–16	11–14

the difference is greater, usually amounting to 6–8 more teeth on each side. The anterior teeth in *S. helenae* exhibit a very noticeable overlap a short distance back from the points. In the majority, the teeth stand out from the head and resemble, in miniature, a small curved fan.

Distribution. This was the most common chaetognath in the plankton at station 1C, 2C and on occasions at 3C. A form widely ranging along the west coast, it was customarily found in water of 34–35$^0/_{00}$. It is significant that its presence at any station was with few exceptions assurance that *S. hispida* would be absent or found there in only minor numbers (Tables I, II and III). The Cedar Key area offered the least favorable environment for this species. It was never taken there at either 3A or 3B stations and in a number of instances it was absent from 3C as well.

Further information concerning the distribution of *S. helenae* along the west coast was obtained from a number of scattered samples taken over a period of several years. Two samples taken 30 and 50 miles southwest of Bradenton Beach, October, 1949, contained *S. helenae* in some abundance. A plankton tow made 12 miles northwest of Key West in July, 1946 contained a few *S. helenae.* Other samples taken in the close vicinity of Key West and in Florida Bay contained no representatives of this species. This information strengthens the evidence that *S. helenae*

will not tolerate indefinitely conditions where the salinity is several parts per thousand below normal, but will be found in high salinity water over the continental shelf along the west coast of Florida. No positive information concerning its offshore distribution in the Gulf is at present available.

Breeding. Evidence for year round breeding of *S. helenae* is seen in the abundance of Stage I individuals in every month. The general distribution of all stages during the year makes it difficult to select periods in which reproduction was accelerated. The large increase in the numbers of young individuals in May and June suggests that this was a time when breeding had increased.

TABLE IX

Stages of maturity for S. helenae
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total
	No. spec.	%	No. spec.	%	No. spec.	%	
1948							
Nov.	181	90	17	9	2	1	200
Dec.	108	87	15	12	1	1	124
1949							
Jan.	231	94	16	6	0	0	247
Feb.	50	62	19	23	12	15	81
Mar.	243	90	23	9	3	1	269
Apr.	53	58	33	36	5	6	91
May	315	88	41	11	2	1	358
June	155	83	27	14	6	3	188
July	21	25	50	59	14	16	85
Aug.	87	94	6	6	0	0	93
Sept.	63	67	31	33	0	0	94
Oct.	29	71	7	17	5	12	41
Nov.	148	92	13	8	0	0	161
Dec.	29	88	4	12	0	0	33
1950							
Jan.	159	73	44	21	14	6	217

Abundance. The abundance of *S. helenae* at the C stations is generally similar to the number of *S. hispida* at the A stations (Tables I, II and III). The optimum location sampled regularly was 1C with 2C next. The largest number per cubic meter found at any one time was 325 on 18 June 1949 at 1C.

Sagitta tenuis Conant (Fig. 4)

Sagitta tenuis Conant, 1896, p. 213; Fowler, 1906, p. 61; Michael, 1911, p. 72, 1919, p. 239; Germain and Joubin, 1916, p. 51. (?); John, 1933, p. 4.
Sagitta friderici Ritter-Zahony, 1911a, p. 19, 1911b, p. 21.

Description. Described by Conant in 1896 from specimens collected June, 1893, Kingston Harbor, Jamaica. Several syntypes have been deposited by him in the

United State National Museum (Cat. No. 4986). A comparison of these specimens with the ones collected on the west coast revealed that they were the same species. Again there is an inadequate description and no figure. The following data were listed in the original description:

Maximum length	Tail segment	Hooks	Anterior teeth	Posterior teeth
5.25 mm.	25 per cent approx.	7–8	4–5	7–10

The greatest discrepancy in the above figures lies in the smaller number of posterior teeth listed by Conant. This is probably not very significant in view of the very small specimens with which he had to work.

These are small slender chaetognaths. The fins are completely set with rays with posterior and caudal fins touching the seminal vesicles in mature individuals. The anterior fins reach forward to the ventral ganglion. The corona is elongate, extending from beyond the eyes more than halfway to the ventral ganglion. The seminal vesicles are slender with a rounded knob on the anterior end when mature. The posterior fins lie more on the tail segment than on the trunk. A line drawn along the edges of each group of anterior teeth would meet medially at an acute to right angle. The collarette is small, evident at the junction of the head and body. The ovaries are short, not usually extending much beyond the posterior fin. The individual eggs are few and rather large in the short ovary. *S. tenuis* can readily be separated from *S. hispida* by the shape of the seminal vesicles and the arrangement of the anterior teeth.

Ritter-Zahony (1910, p. 141) attributes characters described by Conant for *S. tenuis* to *S. bipunctata* and fails to recognize it as a valid species. Michael (1911, p. 73) recognizes *S. tenuis* as a species in good standing. On the other hand, *S. friderici* Ritter-Zahony (1911b) is almost certainly a synonym of *S. tenuis*. The description and figures of *S. friderici* are in complete accord with the specimens from the west coast of Florida, and Conant's *S. tenuis*. Ritter-Zahony obtained his collection from the neighborhood of Porto Grande, Cape Verde Islands.

TABLE X

Measurements of S. tenuis from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
6	25	8	6	15
7	26	7–8	6–7	18–19
8	27	8	7	15

Distribution. The most widely distributed chaetognath on the west coast, it was found on occasion at every station, although somewhat less abundantly at the A stations. It also occurs on the east coast and apparently in greater numbers than on the west coast. Specimens were obtained from Salerno, and St. Augustine, Florida, and Beaufort Inlet,[6] North Carolina.

[6] Beaufort Inlet material obtained through the courtesy of Dr. W. H. Sutcliffe, Jr., Duke Marine Laboratory, Beaufort, N. C.

Breeding. This species appears to breed to some extent throughout the year. Mature forms were found in every month, with the exception of October when very few specimens were captured in any stage.

Abundance. S. tenuis, more than either of the two previously discussed species, shows some seasonal occurrence. It was present in numbers during winter and spring but diminished or disapppeared during the summer and early fall months. This coincides with the period of highest water temperature (Fig. 2) and possibly

TABLE XI

Stages of maturity for S. tenuis
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total no.
	No.	%	No.	%	No.	%	
1948							
Nov.	12	32	18	47	8	21	38
Dec.	60	58	32	31	12	11	104
1949							
Jan.	45	30	63	40	45	30	153
Feb.	20	43	14	30	13	27	47
Mar.	66	38	60	34	49	28	175
Apr.	39	35	53	47	21	18	113
May	0	0	25	58	18	42	43
June	26	51	21	41	4	8	51
July	1	4	16	64	8	32	25
Aug.	0	0	5	50	5	50	10
Sept.	2	8	15	62	7	30	24
Oct.	0	0	3	100	0	0	3
Nov.	7	33	11	52	3	15	21
Dec.	0	0	17	61	11	39	28
1950							
Jan.	3	7	15	35	25	58	43

the high water temperatures affected it adversely. Although widely distributed, it did not occur in as large numbers as did *S. hispida* and *S. helenae.*

Sagitta enflata Grassi (Fig. 4)

Sagitta enflata Grassi, 1881, p. 213; Beraneck, 1895, p. 153; Aida, 1897, p. 15; Doncaster, 1902, p. 210; Fowler, 1906, p. 8; Ritter-Zahony, 1910, p. 139, 1911a, p. 13, 1911b, p. 16; Michael, 1911, p. 28, 1919, p. 242; Bigelow, 1926, p. 334; Burfield and Harvey, 1926, p. 111; John, 1933, p. 1; Tokioka, 1939, p. 126, 1940a, p. 2, 1940b, p. 369, 1942, p. 527; Redfield and Beale, 1940, p. 472; Clarke, Pierce and Bumpus, 1943, p. 221; Thomson, 1948, p. 18, 1947, p. 11; Moore, 1949, p. 25; Davis, 1949, p. 88; King, 1949, p. 119.

Sagitta flaccida Conant, 1896, p. 213; Doncaster, 1902, p. 211.

Sagitta gardineri Doncaster, 1902, p. 212; John, 1933, p. 2.

Sagitta inflata Germain and Joubin, 1916, p. 33.

Description. *S. enflata* is a medium sized, well known and easily recognized chaetognath. The overlapping anterior teeth, the short corona situated on the head, the small knob-like seminal vesicles, together with the flaccid body are features which characterize this species.

Distribution. Along the west coast *S. enflata* occurred seldom or never at the A and B stations and was present in reduced numbers and then only at scattered intervals at 3C (Tables I, II and III). It occurred normally in the samples in which *S. helenae* was found, and if *S. helenae* were absent *S. enflata* was usually absent as well. As was the case for *S. tenuis,* it almost completely disappeared during late summer and early fall. *S. enflata* appears to have little tolerance for either low salinities or persistent high temperatures. In the tropics the ability of *S. enflata* to perform vertical migrations might remove it when necessary from the very warm

TABLE XII

The measurements of S. enflata from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
8–9	15–18	8–9	5–6	8
10–11	17	8–9	5	8
12–13	16–18	8–9	6–7	11
14–15	16–17	8–9	7–9	10–13
16–17	17	8	9	14
18–19	15–17	8	7–9	13–14
20–21	15	9	11	15

surface waters. Moore (1949) states that in the Bermuda area its mean day level was 115 meters.

S. enflata is typically an offshore species. It has a cosmopolitan distribution in all the warmer seas. Ritter-Zahony (1911b) states that its range is between 40°N and 40°S latitude. These limits appear to be generally true as far as the existence of a permanent population is concerned, although there are records of its occurrence farther north (Redfield and Beale, 1940).

Breeding. The data are too scattered to permit valid conclusions concerning the breeding season; however, the presence of Stage I forms at all seasons suggests that the period of reproduction is a long one in this area.

Abundance. Judged on a year round basis this species was fourth in abundance in the west coast area (Tables I, II and III). Usually occurring in numbers of less than 50 per cubic meter, it occasionally exceeded this figure in the winter months.

Krohnitta pacifica (Aida) (Fig. 4)

Krohnitta pacifica Tokioka, 1939, p. 135, 1940a, p. 7, 1942, p. 546; Thomson, p. 22.
Krohnia pacifica Aida, 1897, p. 19; Doncaster, 1902, p. 215; Fowler, 1906, p. 24.
Krohnitta subtilis (partim) Ritter-Zahony, 1910, p. 140, 1911a, p. 44, 1911b, p. 32.
Eukrohnia pacifica Michael, 1911, p. 76.

Description. Ritter-Zahony (1910) established the genus *Krohnitta* and synonymized *K. pacific* under *K. subtilis.* As indicated by Tokioka (1939) and

TABLE XIII

Stages of maturity for S. enflata

Data are included from all stations at which specimens were found

Date	Stage I		Stage II		Stage III		Total no.
	No.	%	No.	%	No.	%	
1948							
Nov.	20	76	3	12	3	12	26
Dec.	79	79	16	16	5	5	100
1949							
Jan.	119	75	20	12	20	12	159
Feb.	115	89	11	9	3	3	129
Mar.	120	74	36	22	7	4	163
Apr.	16	53	5	17	9	30	30
May	81	88	11	12	0	0	92
June	10	77	3	23	0	0	13
July	1	100	0	0	0	0	1
Aug.	2	100	0	0	0	0	2
Sept.	4	100	0	0	0	0	4
Oct.	1	50	1	50	0	0	2
Nov.	82	99	0	0	1	1	83
Dec.	42	93	3	7	0	0	45
1950							
Jan.	42	71	17	29	0	0	59

Thomson (1947) these are separate and clearly defined species. *K. pacifica* is a shorter, broader form with a relatively large ovary extending beyond the border of the posterior fin when mature. The ovary in *K. subtilis* is small, hardly as long as the width of the body.

Distribution. The occurrence of *K. pacifica* along the west coast was sporadic. It was found in some of the fall and winter samples at 1B, 1C and 2B, 2C in small numbers (Tables I, II and III). Only two specimens were taken in the quantita-

TABLE XIV

Measurements of K. pacifica from the west coast of Florida

Length mm.	Tail segment %	Hooks	Teeth
5	28–29	9	11
6	27–28	8–9	12
7	30	9	12

tive sampler in the Cedar Key area. This latter region is clearly the least favorable of the three for this species.

Breeding. Not enough specimens were collected to warrant definite conclusions on the breeding of this species; however, whenever any number were taken, some of them were almost invariably mature. This strongly suggests that the breeding season for *K. pacifica* is a long one in this region. Stage I individuals

were rarely recorded because their minute size (two–three mm.) made selection from the samples difficult and subject to error.

Abundance. It was clearly the least abundant of the chaetognaths collected. The maximum number encountered was 28 per cubic meter in November, 1949 at station 1B. Although it did not appear simultaneously at the Ft. Myers Beach or

TABLE XV

Stages of maturity for K. pacifica
Data are included from all stations
at which specimens were found

Date	Stage I	Stage II	Stage III	Total
	No.	No.	No.	
1948				
Nov.	0	14	44	58
Dec.	0	8	3	11
1949				
Jan.	0	2	2	4
Feb.	0	0	4	4
Mar.	0	1	2	3
Apr.	2	3	3	8
May	0	0	0	0
June	0	0	0	0
July	0	1	0	1
Aug.	0	1	1	2
Sept.	0	4	3	7
Oct.	0	4	12	16
Nov.	1	8	6	15
Dec.	0	1	8	9
1950				
Jan.	0	7	8	15

Bradenton Beach stations, it was captured on 13 occasions in each area and in roughly comparable numbers.

Regeneration in Chaetognaths

An unusual phenomenon was observed in a number of specimens of *S. helenae* and *S. enflata* (26 were taken from the samples). These individuals were found to have lost their heads, together with a short section of the trunk and were in various stages of head regeneration (Fig. 5). This condition was at first attributed to accidental damage encountered in the net; however, as additional specimens were encountered in which various degrees of head regeneration could be observed, it was concluded that this was not an unusual occurrence and that the loss of this section of the body did not necessarily kill the individual. No instances of the re-growth of the tail or posterior end of the body were observed.

Upon the loss of the head the trunk tissues contract tightly together at the severed end. This gives the appearance of the tied end of a sausage casing. The

head begins to form within the constricted end. The eyes appear early, followed by the mouth and finally jaws appear.

Other features which lend interest to this observation are that although many hundreds of *S. setosa* and *S. elegans* from northern latitudes were carefully inspected in other studies, no evidence of regeneration of the head was ever observed. No mention of such phenomena has been recorded in the literature reviewed.

FIGURE 5. Chaetognaths showing various stages of head regeneration. The three specimens on the left are *S. helenae*. The three on the right are *S. enflata*.

DISCUSSION

A comparison of the three base areas shows a considerable similarity between Bases 1 and 2 from the point of view of occurrence and abundance of the five species. The Cedar Key area, with its reduced salinity and the predominance of *S. hispida,* showed somewhat greater differences in its chaetognath fauna from the other two.

The distribution of the chaetognaths along the coast is consistent with the hydrographic conditions operating there. Inshore, there is a very appreciable drift of water northward along the coast (United States Hydrographic Office, Current Charts, Central American Waters). Although the figures vary considerably from month to month, a rough average of 6–8 miles per day would indicate the magnitude of the movement involved. In the Bradenton Beach area this northward counter current may at times exceed 20 miles per day. The fact that the movement of water is parallel to the coast, together with the relatively mild tides in this area, allows salinity and faunal areas paralleling the coast to persist almost indefinitely. At the same time, this northward movement of water is responsible for the wide distribution of the chaetognaths within the respective areas of salinity. It was significant to find the difference in distribution between *S. hispida* on the one hand and *S.*

helenae and *S. enflata* on the other. Along the west coast of Florida the former was characteristically found in the inshore water of low salinity, whereas the two latter species were seldom taken in company with *S. hispida.* The year-round persistence of the difference in the distribution of the species substantiates the lack of lateral mixing which was also indicated by the salinity gradient found along the coast. There is reason to believe that these species might be successfully used as indication of the movement of water from offshore inward or from the inshore outward.

During the period of this study, despite a hurricane which traversed almost the length of the state on August 26–27, 1949 (Latour and Bunting 1949), no unusually high salinity records were obtained at the inshore stations, nor was there any evidence from chaetognath distribution that species occurring offshore had been swept inshore in any numbers. In fact, the reverse appears to have taken place. On 12 September, 16 days following the storm, *S. hispida* was taken at station 1C which was the first time in many months that it had appeared at this station, and *S. helenae* was absent for the first time. *S. hispida* was taken at 2C for the first time the following month.

Additional collections of chaetognaths are needed from points far out into the Gulf and at various depths to allow us to define better the offshore range of such species as *S. helenae, S. tenuis* and *K. subtilis.*

Summary

1. From November, 1948 through January, 1950 quantitative plankton collections were made at monthly intervals from nine stations along the west coast of Florida.

2. Surface water temperatures and salinities were taken at each station. The warmest water temperatures were similar at all stations but the coldest water by several degrees was found at the Cedar Key station (3A).

3. The inshore water at all stations showed reduced salinity. This was correlated with rainfall. The salinity increased from the inshore to the offshore stations.

4. Three maturity stages were described for the chaetognaths based on development of testes and ovaries.

5. The chaetognaths collected were: *S. hispida, S. helenae, S. tenuis, S. enflata, K. pacifica.* One of these, *S. hispida,* exists characteristically in dilute inshore water. *S. helenae* and *S. enflata* were normally absent at the inshore stations where *S. hispida* was abundant. They were most numerous at the C stations where the salinity of the water varied around $35^0/_{00}$. *S. tenuis* appeared to be rather tolerant of fluctuations in salinity although in the Cedar Key area, where the lowest salinities were encountered, it was noticeably scarce at the inshore stations. The catches of *K. pacifica* were too few and scattered to give a good picture of its local distribution. It was more abundant in waters of high salinity than elsewhere.

6. The data indicated that breeding was, with some fluctuations, continuous the year round.

7. *S. hispida* and *S. helenae* were found at all seasons. Increased numbers of Stage I forms appeared in the late spring and summer, resulting in an over-all increase in the population. *S. tenuis* and *S. enflata* were noticeably most abundant during the colder months.

8. A northward movement of the coastal water helps distribute the chaetognaths and apparently does not interfere seriously with the persistence of water of reduced salinity in the inshore areas.

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SOME ASPECTS OF RESPIRATORY METABOLISM DURING METAMORPHOSIS OF NORMAL AND DDT-RESISTANT HOUSE FLIES, MUSCA DOMESTICA L.

BERTRAM SACKTOR

Chemical Corps Medical Laboratories, Army Chemical Center, Maryland

In this study, the respiratory metabolism of two strains of house fly, one of them resistant to DDT, has been investigated in order to ascertain some of the biochemical events associated with developmental processes, and to contribute to our scant knowledge of the mechanism of insect resistance to insecticides.

Inasmuch as both DDT and cyanide are known to inhibit cytochrome oxidase, it seemed worthwhile to compare the effects of cyanide on normal and resistant strains of a single species. Pupae were used in these experiments because measurements of their oxygen consumption could be made without the interference of bodily activity, and also because they provide an opportunity for gaining a better understanding of biochemical events during metamorphosis.

General reviews of the respiratory metabolism of insects during this stage of development have been compiled by Needham (1942) and Wigglesworth (1947). The inhibitory action of cyanide, azide and carbon monoxide on cytochrome oxidase has been studied by Keilin and Hartree (1939) and by others. With insect material, the effects of one or more of these inhibitors have been reported for eggs of the grasshopper, *Melanoplus differentialis* (Bodine and Boell, 1934); for larvae of the codling moth, *Cydia pomenella* (Graham, 1946); and for pupae of *Drosophila melanogaster* (Wolsky, 1938). Collier (1940) and Zukel (1944) studied the inhibition of cytochrome oxidase by phenothiazine, while Sacktor (1949) and Johnston (1950) found that DDT also inhibits this enzyme.

The development of insect resistance to insecticides has become a problem of major economic and, potentially, medical importance. Quayle (1916) and others showed that certain scales had become resistant to cyanide. Cyanide resistance has been reported also for *Drosophila melanogaster* and an aphid, *Aphis gossypii* (Boyce, 1928), as well as for the confused flour beetle, *Tribolium confusum* (Gough, 1939). Screw-worms, *Callitroga americana*, resistant to phenothiazine were found by Knipling (1942). In recent years, reports of house flies and other insects resistant to DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) and other synthetic insecticides have become commonplace. The subject has been reviewed by Babers (1949). Since then, experiments by Sternburg and Kearns (1950) and Sternburg, Kearns and Bruce (1950) revealed that all stages of several DDT-resistant strains of house fly were capable of converting DDT to non-toxic DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), whereas only pupae of a normal strain could metabolize DDT to DDE. In partial agreement with the above results, Perry and Hoskins (1950) found that adults of both a resistant and normal strain could detoxify DDT but that the extent of this detoxification was greater with the resistant strain. Despite these observations, it may still be said that

little is known about the physiological mechanisms concerned in resistance to insecticides, since the mode of action of the compounds themselves is seldom understood.

Methods

The DDT-resistant strain of house flies was derived from the Ellenville line. Details concerning this stock and the rearing procedure have been reported elsewhere (Sacktor, 1951).

Warburg manometers were used for determinations of normal and cyanide inhibited oxygen consumption. Four pupae of a given age were employed per determination. In measuring the normal oxygen consumption the pupae were placed in the main compartment of the flask, the center well containing 0.2 ml. of 0.5 *M* KOH. To determine the consumption during cyanide inhibition, the pupae were placed in the side arm of the vessel. As recommended by Robbie (1946), the center well contained KCN-KOH or $Ca(CN)_2$-$Ca(OH)_2$ mixtures, depending on the experimental concentration of cyanide used. The former mixture was used for determinations of the inhibition with 3×10^{-4} *M* cyanide, the latter with 4×10^{-3} and 5×10^{-2} *M*. When measuring the inhibition with the lowest cyanide concentration, the main compartment of the flask contained 1.0 ml. of *M*/150 phosphate buffer, pH 7.0, and 3×10^{-4} *M* KCN. Nothing was placed in the main compartment when determining the inhibition with higher cyanide concentrations.

The $Ca(CN)_2$-$Ca(OH)_2$ mixtures were prepared according to the procedure of Robbie and Leinfelder (1945). The HCN concentration of these mixtures was determined by the method of Liebig (1851). The experimental HCN concentrations were interpolated or extrapolated from the data given by Robbie (1946).

The flasks were allowed to equilibrate for 30 minutes at 30° C. with a shaking rate of 120 per minute. The oxygen uptake was thereafter recorded every 10 minutes for one hour.

Cytochrome oxidase activity was measured in a Beckman spectrophotometer at 5500 Å. The procedure described in detail in a previous paper (Sacktor, 1951) was followed with but one minor modification: For each determination 5 pupae were homogenized in 5.0 ml. iced distilled water. A 1.0 ml. aliquot of this stock homogenate was diluted five-fold, and paired determinations were made using 0.5 ml. of the diluted homogenate.

To determine the mortality of pupae exposed to cyanide vapor, the pupae were collected from the Warburg flasks immediately after respiration studies. These pupae were then washed, dried and placed in petri dishes. The non-emergence of the flies was used as the death criterion.

Results

The effect of cyanide at various concentrations on the oxygen consumption of normal pupae is shown in Figure 1. Each point on the curves representing the normal oxygen consumption and that of pupae exposed to 0.3×10^{-4} *M* cyanide indicates the average of 12 determinations. The remaining points represent averages of from 4 to 12 determinations each. The per cent of respiration insensitive to cyanide varied with the age of the pupae, as recorded in Table I.

TABLE I

Per cent of pupal respiration insensitive to cyanide

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-2}	3×10^{-4}	4×10^{-3}	5×10^{-2}
0	16	8	6	19	14	8
1	38	36	21	72	69	61
2	45	39	32	67	46	68
3	73	31	10	87	61	37
4	83	24	15	65	25	18

Similar data for the DDT-resistant strain are given in Figure 2 and Table I.

Figure 3 shows the change in cytochrome oxidase activity in both strains of pupae in relation to their development. Each point represents the mean and its standard error for 14 to 26 determinations.

In Table II is shown for both strains of pupae the mortality due to exposure to different cyanide concentrations. These results were obtained by combining those pupae, 16 to 48 in number, used under each experimental condition.

TABLE II

Per cent mortality of pupae exposed to cyanide vapor

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-2}	3×10^{-4}	4×10^{-3}	5×10^{-2}
0	94	100	100	95	100	100
1	25	92	100	9	69	100
2	36	100	100	22	100	100
3	75	100	100	22	88	100
4	88	100	100	29	96	100

DISCUSSION

Oxygen consumption

The oxygen consumption of normal and resistant house fly pupae exhibited the characteristic U-shaped curve during their development. These results are in agreement with a multitude of other investigations with holometabolic insects (Haub and Hitchcock, 1941, with Phormia; Frew, 1929, with Calliphora; Wolsky, 1938, with *Drosophila melanogaster;* Dobzhansky and Poulson, 1935, with *D. pseudoobscura;* Ludwig, 1931, with Popillia; Lindgrem, 1935, with Tribolium; and others).

Figures 1 and 2 reveal that the oxygen consumption of the white pre-pupae (zero age) was considerably higher then than it was immediately prior to the

emergence of the adults. This is an apparent disagreement with some other investigations. Oxygen consumption of the early stages, however, was measured in these experiments within one-half hour after puparium formation. This is as much as 10 hours earlier than has been reported by some other investigators. It is

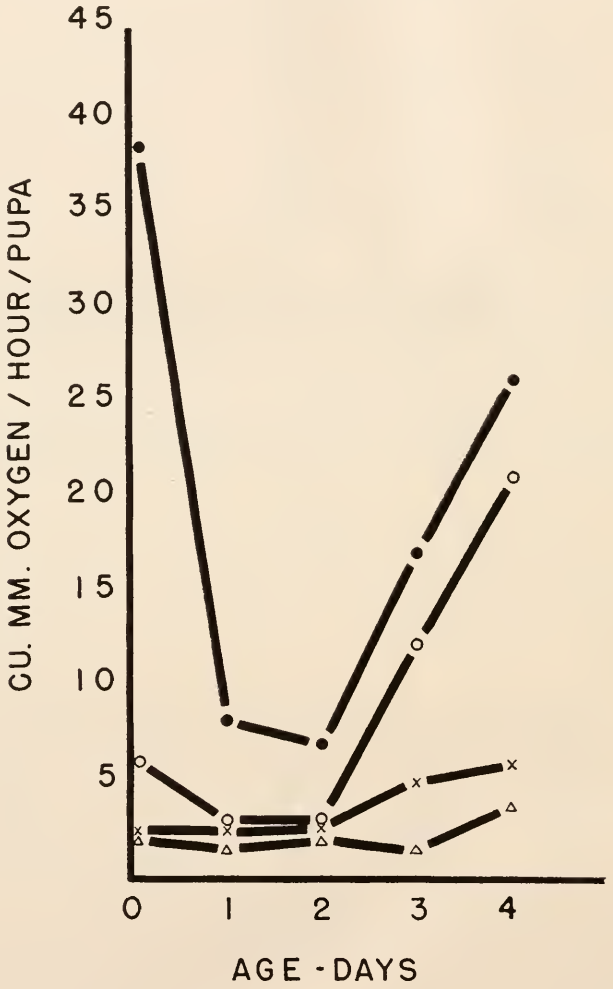

FIGURE 1. The effect of cyanide at various concentrations on the oxygen consumption of normal house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M*, cyanide.

thus evident that oxygen consumption must decrease rapidly at this critical period. This interpretation is in agreement with measurements of oxygen consumption in *Drosophila pseudoobscura* by Dobzhansky and Poulson (1935), and with the unpublished data of Bodenstein and Sacktor on larval and pupal cytochrome oxidase in *D. virilis,* and of Levenbook (1950) on respiration in Calliphora.

The significance of the U-shaped curve is not fully understood. Gaarder (1918) has shown that the fall in oxygen uptake is not due to a reduction in the oxygen tension in the tissues. As pointed out by Needham (1942), the idea "that it (the change in oxygen uptake) represents the metabolic consequences of

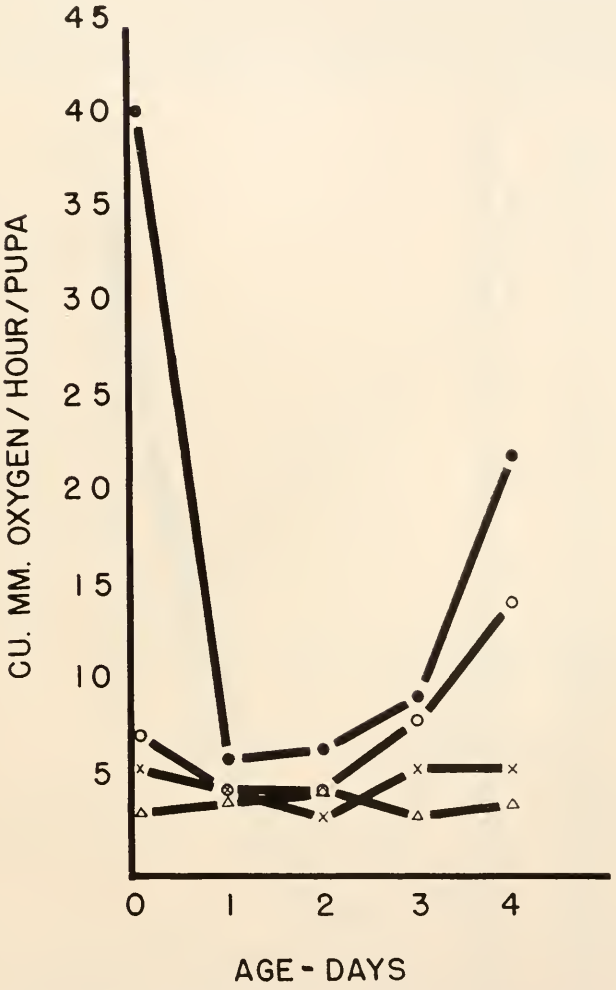

FIGURE 2. The effect of cyanide at various concentrations on the oxygen consumption of DDT-resistant house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M* cyanide.

complete histolysis followed by new tissue differentiation . . . cannot be true." Such a view, which was once widely accepted, is not consistent with the histological data found by Dobzhansky and Poulson (1935) for Drosophila pupae. Agrell (1947) assumed that the U-shaped course of respiration is, to a certain degree, connected with variations in the activity of the dehydrogenase systems. Wolsky

(1938) attributed the changes in oxygen consumption to the quantity or activity of the cytochrome system. The results of the present study support Wolsky's interpretation in part, but point also to additional complicating factors.

Cytochrome oxidase

The cytochrome oxidase activity of both strains changes during pupal development. The activity follows a U-shaped curve (Fig. 3), which has, in general, a

FIGURE 3. The change in cytochrome oxidase activity in both strains of house fly in relation to their pupal development. Solid circles represent normal strain; open circles represent DDT-resistant strain.

similar appearance to that of oxygen consumption *in vivo*. The relationship of respiration to cytochrome oxidase activity is shown in Figure 4 (Boell, 1945, found a like comparison with developing embryos of *Amblystoma punctatum*).

The broken line drawn through the origin is based on the assumption of 1 : 1 correspondence between oxygen uptake and cytochrome oxidase activity. In plotting these data, the total measured oxygen consumption was corrected by subtracting the average cyanide insensitive respiration of 2.0 cu. mm./hr./pupa for the normal strain and 4.0 cu. mm./hr./pupa for the resistant strain. The graph shows that an approximately linear relationship exists between cyanide sensitive respiration and cytochrome oxidase activity during pupal development for 1–4 days of age.

It will be noted from Figure 4 that some of the points reveal that there is slightly more cyanide sensitive respiration than is accountable by cytochrome

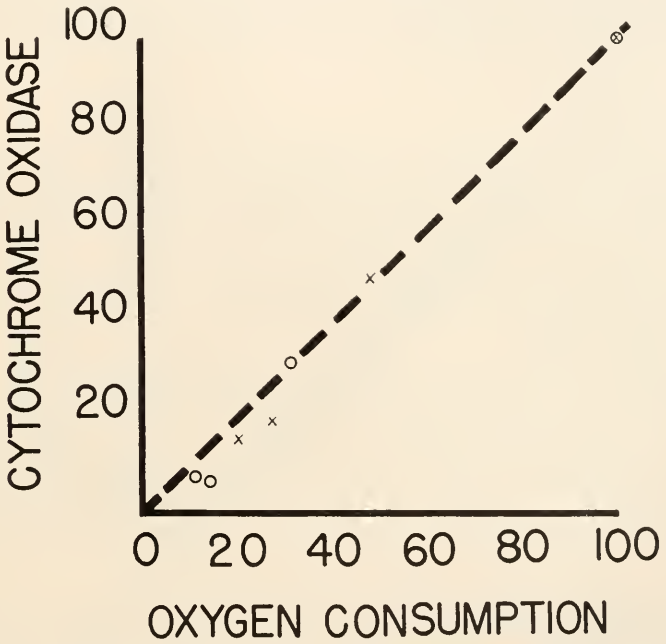

FIGURE 4. The relationship of oxygen consumption to cytochrome oxidase activity. All numbers in per cent of the highest O_2 consumption and oxidase activity observed. Crosses represent normal strain; open circles represent DDT-resistant strain. For further details see text.

oxidase activity. An examination of Figures 1 and 2 shows that these points correspond to pupae one and two days old. Further, the data obtained for zero age pupae are not included in the curve of Figure 4. These data (compare Figs. 1 and 3) indicate that there is at this stage a great excess of cyanide sensitive respiration not accounted for by cytochrome oxidase, and thus suggest that other cyanide sensitive systems are contributing to the total oxygen consumption. Such systems might involve tyrosinase or catalase, which are cyanide sensitive enzymes likely to be present in animal tissues. But from the existing evidence (Fink, 1930; Williams, 1936) there seems to be little correlation between catalase content of insects and the intensity of their respiration. Dennell (1947), however, related tyrosinase activity to puparium formation. In his review of tyrosinase in insects,

Sussman (1949) reported that tyrosinase activity increased until pupation, whereupon there was a rapid decrease.

Thus, the present experiments provide some evidence partially substantiating Wolsky's hypothesis, that changes in oxygen consumption during metamorphosis are related to the activity of cytochrome oxidase. But there are other factors here, in addition to the cytochrome system, which contribute to the total respiration. These are a cyanide insensitive system (which will be discussed later) and another cyanide sensitive system, perhaps tyrosinase. The latter is significant mainly in the early stages of pupal development.

The factors that cause changes in the cytochrome oxidase activity are not known, and additional experiments, some of which are underway, are necessary in order to ascertain the significance of these changes.

Inhibition by cyanide

Cyanide inhibition experiments (Commoner, 1940; Robbie, 1949; and others) reveal considerable variation among organisms in regard to their cyanide sensitive and insensitive systems. Runnström (1930) with the sea urchin and Bodine and Boell (1934) with the grasshopper showed that cyanide had various effects on different embryonic stages. It is apparent from Figures 1 and 2 and Table I that in house fly pupae the magnitude of inhibition by cyanide is dependent on the the developmental stage.

Upon complete inhibition of the cyanide sensitive respiration, the cyanide stable respiration (in cu. mm./hr./pupa) was approximately constant throughout development. As seen from Figure 5, the per cent inhibition was therefore found to be dependent upon the original cyanide-free rate of respiration. Only one deviation was observed, *i.e.*, with 4 day old pupae of the normal strain. As can be seen from Figure 1, this deviation can best be explained by the apparent failure of 5×10^{-2} *M* cyanide to inhibit completely the cyanide sensitive respiration of that stage.

It is also evident that the effects of submaximal cyanide concentrations depended upon the developmental stage of the pupae, for inhibition at a given concentration of cyanide decreased markedly in the later stages. Similar results were obtained by Wolsky (1938), with carbon monoxide and Drosophila pupae. In general terms, he proposed two explanations, namely; "(1) there may be changes in the physical-chemical properties of the medium in which the Warburg-Keilin system is reacting, affecting the velocity constants of the reactions or the solubility of the gases; (2) there may be a qualitative change in the Warburg-Keilin system, which alters the velocity constants of its reactions, so that it reacts more readily with O_2 and less readily with CO." He also noted that the work of Szorenyi and Tschepinoga (1936), who reported that in trained muscles the oxygen consumption increased and at the same time the respiration became more resistant to cyanide.

This partial inhibition by cyanide and carbon monoxide is of considerable interest and may reveal certain properties of the Warburg-Keilin system. Unfortunately the possible explanations are based partly upon conjecture, but a discussion of them may be worthwhile in directing future experimentation. Cook *et al.* (1931) and Cook and Haldane (1931) showed that with bacteria, at a given level

of cyanide or carbon monoxide, the sensitivity of the respiration was dependent on the nature of the substrate. They observed that the affinity constant (K = affinity of oxidase for O_2/affinity of oxidase for CO) with glucose was approximately 8 ×, 4 × and 2 × that when formate, succinate or lactate, respectively, were used. A similar effect was found by Ogston and Green (1935) for the respiration of yeast in various substrates. At a given cyanide level, the greatest oxygen consumption was with glucose as the substrate. The consumption decreased, in this order, when glycerophosphate, lactate, hexosediphosphate and hexosemonophosphate were used. There is, therefore, evidence that the cyanide

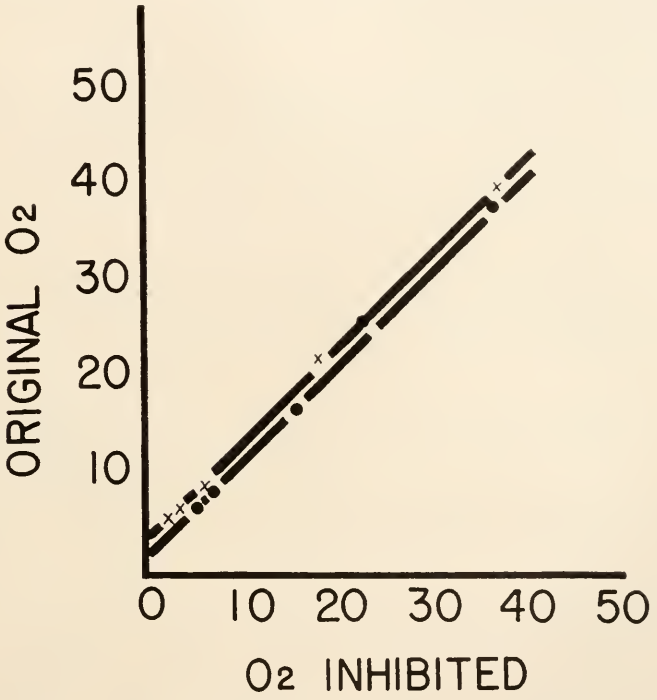

FIGURE 5. Amount of oxygen inhibited as a function of the original oxygen consumption. Ordinate, normal O_2 consumption in cu. mm./hr./pupa. Abscissa, amount of O_2 in cu. mm./hr./pupa inhibited by cyanide. Crosses represent DDT-resistant strain; closed circles represent normal strain. For further explanation see text.

(or carbon monoxide) sensitivity of the oxidation of various substrates is a function of the relative capacity of their specific dehydrogenases. Some dehydrogenases appear to require a greater degree of activity on the part of the oxidase, thus rendering the respiratory rate more sensitive to the decrease in oxidase activity by inhibitors.

During metamorphosis the developing insect utilizes reserve oxidizable substances. In many cases glycogen appears to be utilized throughout metamorphosis (for more details see Rockstein, 1950). As shown by Ludwig and Rothstein (1949) in the Japanese beetle, *Popillia japonica*, glycogen decreases most rapidly

during the first days of pupal life. Glucose also may serve as a substrate during the pupal stage. Evans (1932) found that the glucose content of the blowfly, *Lucilia sericata,* falls in young pupae but remains fairly constant during the later stages. A continuous utilization of glucose during metamorphosis occurred in the mealworm, *Tenebrio molitor,* (Evans, 1934). Courtois-Drilhon (1931), Crescitelli and Taylor (1935) and Ludwig and Rothstein (1949), however, have shown an increase in glucose during the prepupal and pupal period in several species of Lepidoptera and the Japanese beetle. This increase in glucose content is attributed to its conversion from glycogen. There are considerable differences in the utilization of fat during metamorphosis of insects. In general, fats seem to be used primarily in the late stages of development (Frew, 1929; Evans, 1934; and Ludwig and Rothstein, 1949).

The above data reveal that the substrates metabolized during metamorphosis vary according to the developmental stage and with the species studied. In general, carbohydrates are used primarily during the early stages whereas fats are consumed later. Since there is evidence that sensitivity to cyanide is dependent upon the nature of the substrate, the differences found in the effects of submaximal cyanide concentrations on developing house fly pupae may perhaps be related to changes in the substrate being metabolized. Although this inference is supported by analogy with facts determined in studies of other organisms, investigations suitable for testing this hypothesis are yet to be made with the house fly.

Another explanation, although related to the above, of the differences in effects of submaximal cyanide concentrations is the possibility that partial cyanide inhibition may cause a change in the hydrogen donators. Such alterations may even result in a stimulation of oxygen consumption. This possibility is excellently discussed by McElroy (1947) and for details it is recommended that reference be made to his paper. He has shown that the oxygen consuming reactions exhibited by normal tissues may be entirely different from those which maintain the residual respiration during partial inhibition.

Cyanide, in addition to inhibiting cytochrome oxidase, may also react with other metabolic participants and thus have an effect on the oxygen consumption *in vivo.* As shown by Marshall and Rosenfeld (1934), cyanide combines with aldehydic and ketonic substrates to form cyanohydrins. Recently, Lehninger (1950) reviewed the role of metal ions in enzyme systems. Since several heavy metals may react with cyanide, various enzymatic processes in the total respiratory metabolism may be altered. These alternatives should be kept in mind in considering the significance of the present results.

Cyanide insensitive respiration

Upon apparently complete inhibition of the cyanide sensitive respiration, there still remained a residual oxygen consumption. It is evident from Figures 1 and 2 that the cyanide insensitive respiration is relatively constant throughout metamorphosis, although different for each strain. In the normal strain the average cyanide insensitive oxygen consumption was 2.0 cu. mm./hr./pupa, whereas for the resistant strain it was twice as great. In Figure 5, by plotting original oxygen consumption against cyanide inhibited respiration, a line is obtained whose intercept on the ordinate represents the true value of the cyanide stable respiration.

The experimental values (Figs. 1 and 2) agree with the value obtained by such treatment of the data.

Although there are several enzymes, including cytochrome *b*, which are capable of reacting with oxygen and are not inhibited by cyanide, only for one of these, the "yellow enzyme" of Warburg and Christian (1932), are there sufficient data to estimate its significance in relation to the total respiration. The substrates undergoing oxidation through this enzyme are the hexosephosphates, citrate, glucose, and, to some extent, malate. Gourevitch (1937) reported a definite relationship in mammalian tissues between the quantity of flavin and the amount of cyanide insensitive respiration. Groen and Schuyl (1938) fed rats a flavin-free diet and found that the loss of flavin from the liver was accompanied by a corresponding reduction in the cyanide insensitive respiration. Normal and treated kidney, however, contained the same amount of flavin and the cyanide stable oxygen consumption was the same. Further evidence is found in the experiments of Pett (1936), who showed that when yeast is cultured in a medium containing cyanide, its flavin content is doubled and that correspondingly the cyanide insensitive respiration is also doubled. Thus, it is indicated that the absolute value of the cyanide stable respiration is directly related to the flavoprotein content of the tissue.

Although, as yet, flavin determinations have not been made on house fly pupae, the fact that the DDT-resistant strain has a greater cyanide insensitive respiration than the normal strain suggests the desirability of such a comparative study.

Comparison of the two strains.

The normal oxygen consumption of both strains of pupae during metamorphosis was approximately the same (Figs. 1 and 2).

It has been shown in Figure 3 that in all stages of development, except immediately before emergence of the adults, the pupae of the DDT-resistant strain have less cytochrome oxidase than the normal strain. At zero age the normal strain had approximately 3.5 times as much oxidase. Pupae one, two and three days old had 2.2 times, 2.4 times and 1.4 times as much oxidase, respectively. This suggests that in the resistant pupae a larger portion of the normal oxygen consumption passes through other respiratory systems, as is in fact demonstrated by the data given in Figures 1, 2 and 5, which reveal that the DDT-resistant pupae have twice the cyanide insensitive respiration of the normal strain.

As shown in Table I, oxygen consumption was inhibited by a given cyanide concentration to a greater extent in the normal strain than in the resistant strain. These results may be related to the finding that, as shown in Table II, the DDT-resistant pupae exhibit resistance to this poison.

The data in Table II also reveal that the lethal effect of a given cyanide concentration is dependent on the stage of development. In the normal strain, pupae one and two days old are least susceptible. This is in agreement with the fact that a larger portion of the normal oxygen consumption of these pupae is through the cyanide insensitive system. Of particular interest is the observation that, at a cyanide concentration of 3×10^{-4} *M*, 3 and 4 day old normal strain pupae mostly succumbed, whereas DDT-resistant pupae of the same age mostly survived. At this cyanide concentration the extent of inhibition in both strains was of the same

small magnitude. The possible mechanisms of this difference in survival of the two strains at these ages are speculative. They may be due to: (1) the dependence upon the cytochrome oxidase system for completion of certain developmental processes (Wolsky, 1937) which are stopped by the partial inhibition of the enzyme in the normal strain, whereas in the resistant strain, although the oxidase is also partially inhibited, these processes can proceed by the utilization of the flavin system; (2) more readily reversible inhibition of cytochrome oxidase in the resistant strain; (3) detoxification of cyanide by the resistant strain after dissociation of the inhibitor-enzyme complex. At this moment, we have no means of deciding which of these possible mechanisms, or combination of them, is actually employed.

It should also be noted that the mechanism of cyanide resistance in pupae may be different from that of DDT-resistance in adults. It was shown by Sacktor (1951) that DDT-resistant adults had approximately 50 per cent more oxidase than the normal strain. This suggests that one of the explanations for the resistance of adult house flies to DDT may be the greater oxidase activity, which will permit the continuance of essential physiological functions despite partial inhibition. In contrast, pupae may utilize a cyanide insensitive system for continuance of these functions. Further, the results of Sternburg and Kearns (1950), Sternburg, Kearns and Bruce (1950) and Perry and Hoskins (1950) in regard to the degradation of DDT by resistant strains must be considered. At present the mechanism of detoxification, or the possible role of DDE in preventing lethal effects of DDT is not known, and it is even possible that cytochrome oxidase is concerned in these mechanisms.

The present study suggests that DDT-resistance may be correlated with ability to maintain respiration in the face of partial inhibition of cytochrome oxidase. At the same time, the results draw attention to the complexity of the mechanisms concerned. These will require further investigation from the several other points of view noted above before a satisfactory interpretation can be reached.

Summary

1. Oxygen consumption follows a U-shaped curve during metamorphosis of normal and DDT-resistant strains of house flies. It is of the same order of magnitude in both strains.

2. Cytochrome oxidase activity during metamorphosis also follows a U-shaped curve. There is evidence that changes in oxygen consumption during this process are, in some respects, related to the activity of the oxidase.

3. A cyanide insensitive system, possibly flavin, and another cyanide sensitive system, probably tyrosinase, contribute to the total oxygen consumption. The latter system apparently contributes mainly during the early stages of metamorphosis.

4. The cyanide insensitive respiration of both strains remains relatively constant throughout development. The resistant strain has twice the cyanide insensitive respiration of the normal strain.

5. The DDT-resistant pupae have less cytochrome oxidase activity than normal pupae at all stages except immediately prior to emergence of the adult.

6. The pupae of DDT-resistant flies exhibit resistance to cyanide.

7. The effects of cyanide vary, depending on the developmental stage of the

pupae. A given concentration of the inhibitor produces different degrees of inhibition and mortality in pupae of different age. One of the possible explanations may be a change in the substrate being metabolized. Other possibilities are mentioned.

8. The possible mechanisms of resistance of pupae to cyanide are discussed. These may be: (a) a by-pass of the cytochrome system; (b) a difference in reversibility of enzyme-inhibitor complex or; (c) a detoxification of inhibitor. It is suggested that DDT-resistance may depend, in part, on similar factors.

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STUDIES OF THE DIGESTIVE TRACTS AND THE DIGESTIVE ENZYMES OF THE GOLDFISH, CARASSIUS AURATUS (LINNAEUS) AND THE LARGEMOUTH BLACK BASS, MICROPTERUS SALMOIDES (LACÉPÈDE) [1]

DAYA SHANKAR SARBAHI

203 Ganga Prasad Road, Lucknow, India

A study of largemouth black bass, *Micropterus salmoides* (Lacépède), a carnivorous species, and goldfish, *Carassius auratus* (Linnaeus), an herbivorous species, was undertaken to determine the digestive enzymes secreted by the various regions of the digestive tracts and the associated glands, and to find the sites of their action. This study not only determined the type of foods that can be utilized, but also gave an idea of the relationship of digestive enzymes to different types of alimentary structures.

Previous Studies

Insofar as is known to the writer, no attempt has ever been made to study the digestive enzymes in goldfish and largemouth black bass. Markus (1932), however, determined the extent to which temperature changes influence food consumption in largemouth black bass.

A history of early investigations of digestion in fishes was given by Sullivan (1908). The earliest critical work mentioned was done by Spallanzani in 1783.

Sullivan studied the physiology of the digestive tract of elasmobranchs and found that the mucous membrane of the oesophagus was without glands; the stomachic sac secreted pepsin and hydrochloric acid; the gastric juice did not digest chitin; the mid-intestine and spiral valve had no digestive activity, but activated the pancreas, the latter having the greatest activating power; the pancreas secreted trypsinogen as a rule, but also secreted trypsin and in addition produced amylopsin and lipase; the rectal gland had no digestive activity.

Kingsley (1917) reported that gastric glands extended up into the oesophagus in sturgeons.

Bodansky and Rose (1922) made a preliminary study on digestion in certain elasmobranchs and teleosts and found that coagulated egg albumen was digested very slowly by the proteolytic enzymes of fishes. From extracts of the pyloric caeca of red snapper (*Lutjanus aya*), they obtained trypsin, pepsin, rennin, amylopsin, and lipase (weak). Invertase was present to a very slight extent; inulinase, maltase, and lactase were altogether absent.

Kenyon (1925) reviewed the works of earlier investigators and studied enzymes in bluegill, other fishes, amphibians, reptiles, and mammals. Erepsin was present in as great abundance in the intestinal mucosa of fishes, amphibians, and reptiles

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as in the intestinal mucosa of dog; coagulated egg albumen was apparently digested equally well by the enzymes of fishes, amphibians, reptiles, and mammals; amylase was generally present in very small quantity in extracts of mucosa from the entire alimentary tract of fishes, amphibians, and reptiles. Invertase was found to occur in the intestinal mucosa of the bluegill.

Babkin (1927) found that extract of the intestinal mucous membrane of a top-minnow (*Fundulus heteroclitus*), a fish without a stomach, contained amylase, lipase, enterokinase, and erepsin. The bile from the gall bladder contained amylase, protrypsin, and prosteapsin.

Vonk (1927) found amylase and maltase in the pancreas of carp (*Cyprinus carpio*), pike (sp.), and dogfish (sp.).

MacKay (1929) studied the digestive system of the eelpout (*Zoarces anguillaris*), and found a strong lipase, a weak amylase, and a very weak protease in the mucous membrane of the stomach. A stronger amylase, a weaker lipase, and no protease were present in a mucous membrane of the duodenum. The juice taken from the stomach and duodenum showed a marked proteolytic action as well as an amylolytic and lipolytic action.

Chesley (1934) studied the concentrations of proteases, amylase, and lipase in certain marine fishes. He found that the digestive enzymes paralleled, in quantity, the general activity of the fish; this was especially true of trypsin but not of pepsin; lipase was the least abundant in the fishes that store large amounts of fat. The compact pancreas was the center of enzyme production, while the diffused pancreas produced only a small portion of the enzymes poured into the enteron. When the pancreas was not highly organized, the pyloric caeca seemed to supplant it partially in enzyme secretion.

Battle (1935) studied the digestive enzymes in the herring (*Clupea harengus*), and found pepsin in the extract of the mucosa of the pyloric caeca. Presence of weak lipolytic and amylolytic ferments was indicated in the gastric juice, and strong ones in extracts from the pyloric, caecal and intestinal mucosae.

Bayliss (1935) studied digestion in plaice (*Pleuronectes platessa*), and found that the mucous membrane of the intestine contains trypsin and erepsin; the liver and the wall of the gall bladder were more potent sources of trypsin than was the intestine; the former contained, also, a cathepsin, and the latter an erepsin. The mucous membrane of the intestine, the wall of the gall bladder, and the liver all contained lipase and amylase; the liver was a far more potent source of lipase than either the gall bladder or the intestine.

Digestive Tracts and Associated Glands

The digestive tract of goldfish is an elongated, relatively undifferentiated tube, coiling repeatedly and filling up the greater part of the abdominal cavity. A true stomach, comparable to that of the largemouth black bass is absent, and its place is taken by an enlargement of the intestine, which for convenience is designated as the intestinal bulb. The digestive tract could thus be separated into *mouth, buccal cavity, pharynx, oesophagus, intestinal bulb, anterior intestine,* and *rectum* (Figs. 1 and 2). The exact boundaries of the divisions of the intestine are not well-marked, but are indicated either by variations in calibre, by changes in the character of their epithelial lining, or by valve-like structures at the junctions of the adjoining regions (Fig. 2).

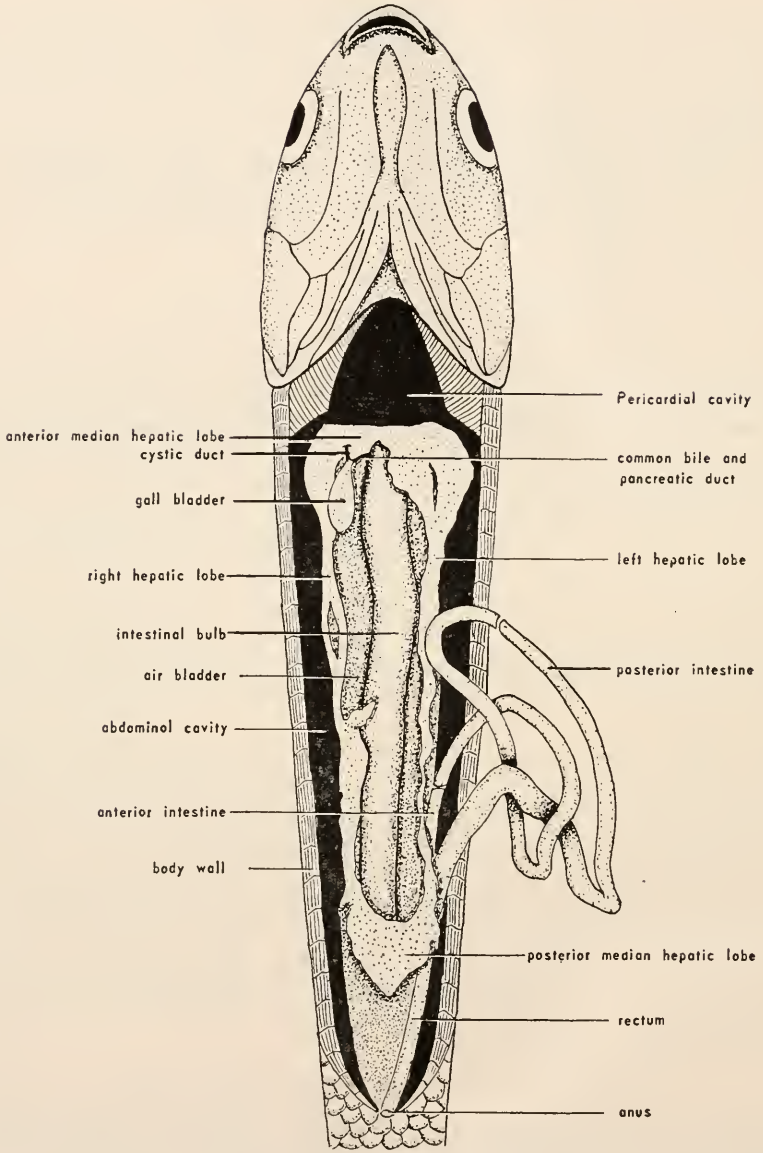

FIGURE 1. Ventral view of the goldfish with body wall and adipose tissue removed to show the viscera (breaks in the coil indicate portions of digestive tract omitted for the sake of brevity).

The *oesophagus* is a short tubular structure, dorsal to the pericardial cavity. The mucous membrane lining the wall is raised into 6 prominent longitudinal folds (Fig. 2). On entering the abdominal cavity, the digestive tract expands to form the intestinal bulb. Externally there is no well-marked indication of the passage of the oesophagus into the intestinal bulb except for an appreciable increase in the

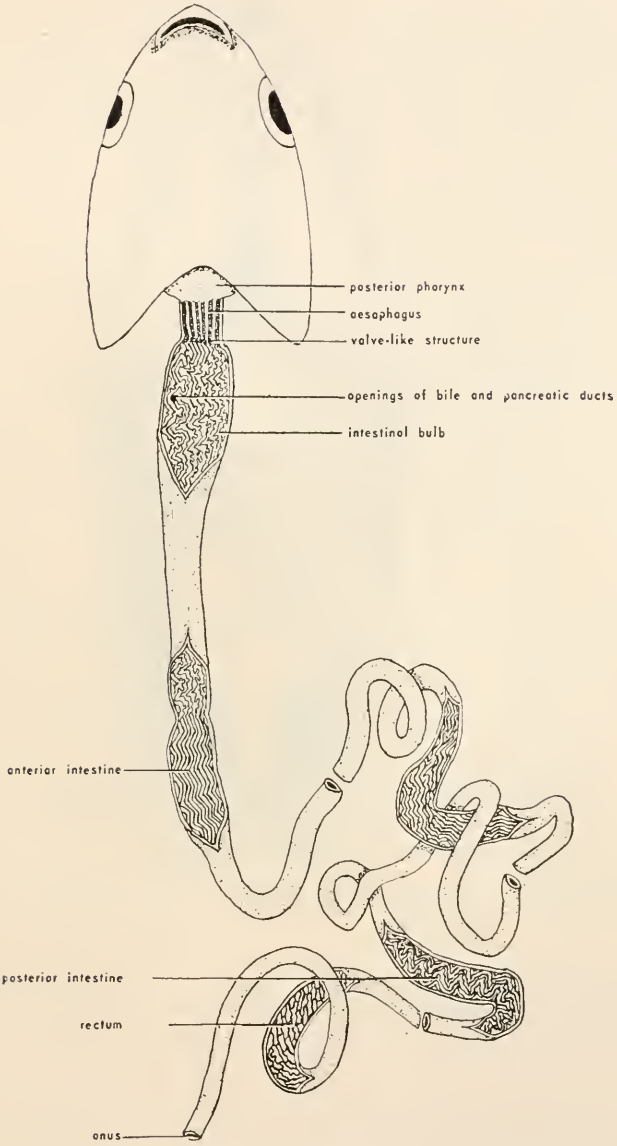

FIGURE 2. Dissection of digestive tract of goldfish to show the characteristics of the mucosal lining of different regions (breaks in the coil indicate portions of digestive tract omitted for the sake of brevity).

diameter of the latter. Internally the two are separated by a valve-like structure (Fig. 2).

The *intestinal bulb* lies dorsal to the coils of the intestine proper and ventral to the air bladder. The bulb is a more or less thickened straight tube, and extends almost to the posterior end of the abdominal cavity. The mucous membrane that

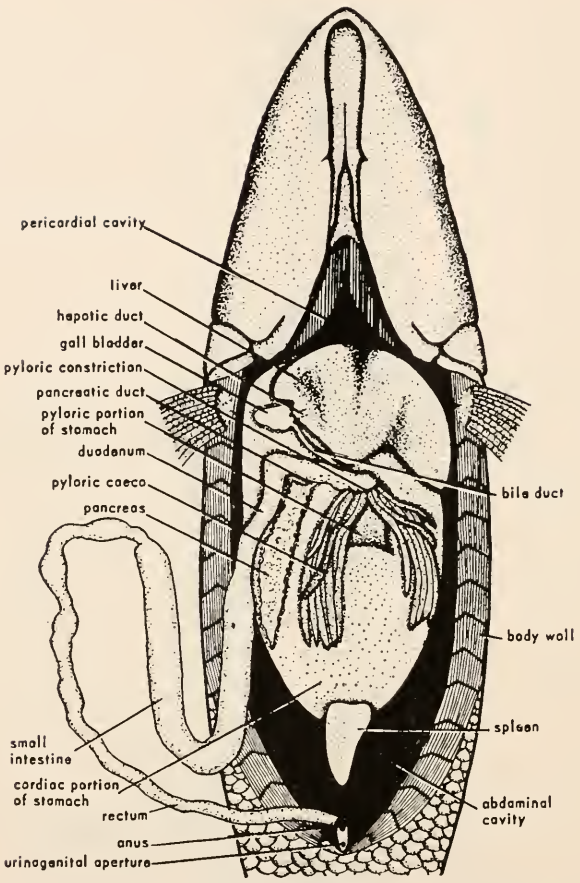

FIGURE 3. Ventral view of the largemouth black bass with body wall and adipose tissue removed to show the digestive tract.

lines the wall is raised into longitudinally disposed folds presenting a somewhat zig-zag pattern (Fig. 2). Posteriorly, the intestinal bulb curves to the left with a slight constriction and passes into the intestine. The pyloric caeca are absent in this species.

The remaining part of the elongated digestive tract behind the intestinal bulb is an elongated, comparatively thin-walled tube, more or less uniform in diameter and disposed into a number of intermixed coils, which fill the entire ventral portion of the abdominal cavity.

Two regions of the intestine proper are distinguished by the disposition of folds of the mucous membrane. The folds present a zig-zag pattern, which in the *anterior intestine* is composed of straight lines and angles, while in the *posterior intestine* it is produced by convoluted lines (Fig. 2).

The *rectum* is a relatively short tube, and is distinguished from the intestine by the presence of obliquely transverse folds in the mucous membrane. These anastamose at intervals (Fig. 2).

The *liver* (Fig. 1) is an elongated mass, consisting of narrow *right* and *left hepatic lobes*, extending on each side of the intestinal bulb. The two lobes join at their anterior and posterior ends to form the *anterior median* and *posterior median hepatic lobes*. The anterior median lobe is attached to the posterior face of the transverse septum separating the pericardial and abdominal cavities. The posterior median lobe is more or less crescentic in shape and extends beyond the limit of the bulb.

Wedged in between the right lobe of the liver and the intestinal bulb is the *gall bladder*.

The pancreas is apparently absent but is probably present in a diffuse mass imbedded in the substance of the liver as was found from a histological study (Sarbahi, 1940) in a closely related species, the Indian carp or rohu (*Labeo rohita*). The liver and the pancreas form the so-called hepato-pancreas. The bile and pancreatic ducts open on the roof of the intestinal bulb a short distance behind the valve-like structure separating the bulb from the oesophagus (Fig. 2).

The digestive tract of largemouth black bass could be differentiated into the *mouth*, the *buccal cavity*, the *oesophagus*, the *stomach*, the *duodenum*, the *small intestine*, and the *rectum* (Fig. 3).

The *oesophagus* is a short tubular structure and lies dorsal to the pericardial cavity.

The *stomach* could be differentiated into a *cardiac* portion and a *pyloric* portion. The cardiac portion, ovoid in shape and highly muscular, when distended with food fills a greater part of the abdominal cavity. The pyloric portion, a short muscular tube, branches off from the mid-ventral surface of the cardiac portion and passes into the duodenum at the pyloric constriction.

The *duodenum* is comparatively narrow. The bile and pancreatic ducts and 21 to 29 digitiform tubules (*pyloric caeca*) open into its anterior end immediately behind the pyloric constriction. The duodenum continues posteriorly into a relatively broad and straight tube, the *small intestine*. The small intestine passes into the narrow thin-walled *rectum*, which opens to the exterior through the anus.

The *liver* is a massive structure. Its anterior margin is crescentic and the posterior margin is provided with a pair of notches. These notches incompletely mark the liver into three lobes, which could be called the *right, median,* and *left lobes*. The *gall bladder* lies dorsal to the median lobe in close proximity to the left lobe.

The *pancreas*, a narrow elongated mass of tissue underlying the cardiac portion of the stomach, runs along the inner wall of the duodenum and is held in position by a mesentery attached distally to the duodenum and proximally to the cardiac portion of the stomach.

Materials and Methods

Preparation of tissue extracts and extracts of juices from the lumen of tracts

Glycerol extracts were prepared from the tissues of hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine of goldfish. Similar extracts were prepared from the liver, pancreas, pyloric caeca, oesophagus, stomach, duodenum, and small intestine of largemouth black bass.

The juices and lumen content from the intestinal bulb, anterior intestine, and posterior intestine of goldfish and from the stomach, duodenum, and small intestine of largemouth black bass were tested to determine the site of action of the various enzymes. After preliminary analyses, extracts from 13 goldfish and 7 largemouth black bass were used to establish fully the presence or absence of each enzyme in question. Each analysis was repeated two or more times on each sample. The size of the goldfish ranged from 13.5 to 20.7 cm. in total length and from 31 to 96 grams in weight. The size of the largemouth black bass ranged from 28 to 31 cm. in total length and from 369 to 454 grams in weight.

Freshly killed fish were immediately dissected under distilled water. The hepato-pancreas in goldfish and the liver, pancreas, and pyloric caeca in largemouth black bass were cleared of the mesentery and surrounding adipose tissue. They were then removed and thoroughly washed in distilled water, and were placed in 50 per cent glycerine covered with toluene for immediate trituration.

The digestive tracts of both the fishes were then ligated at the commencement of the several divisions to prevent passage of the contents from one division into another while being cleaned of the mesentery, and surrounding adipose tissue. Subsequently they were thoroughly washed, removed, wiped dry with filter paper, and cut into the several divisions as previously indicated. Each division was split open by a longitudinal slit, its contents were removed, and the lining was thoroughly washed in distilled water. The contents and washings constituted the sample from that division. If the lumen of any region was empty, it was customary to wash it with distilled water and to analyze the rinsing for the enzymes.

Tissue extracts from the goldfish were obtained by using the oesophagus, intestinal bulb, anterior intestine, and posterior intestine. In the largemouth black bass, tissue extracts were obtained from the mucosae of the oesophagus, stomach, duodenum, and small intestine. These were removed by scraping with a knife. Tissues of entire divisions were triturated in a mortar containing 25 to 50 milliliters of 50 per cent glycerol. Glycerol suspensions of the hepato-pancreas of goldfish and of the liver, pancreas, and pyloric caeca of largemouth black bass were prepared in a similar manner.

Water extracts of the juices or rinsings from various regions of the tracts and the finely ground tissue suspensions in glycerine of the two fishes were kept separately in 50 milliliter Erlenmeyer flasks away from direct sunlight for extraction of enzymes. The extraction was carried out at a temperature ranging from 18 to 22° C., with intermittent mechanical shaking.

The extracts of juices were strained or filtered after 24 to 48 hours; tissue extracts were strained through several thicknesses of cheesecloth after four to eight days. The interval permitted the extractions of enzymes, if present, in sufficient quantities to permit their detection by standard chemical methods.

Toluene was used throughout as a preservative and was added in sufficient quantity to form a layer one-eighth inch in thickness over the extract.

Tests of each extract were run for pepsin, trypsin, rennin, amylase, maltase, lactase, invertase, and lipase. Control samples, which contained an equal quantity of the extract, were boiled to destroy the enzymes and then were given the same treatment as the test samples of each analysis.

Digestive Enzymes

Pepsin

In this work, pepsin was considered to be the proteolytic enzyme which under strongly acid conditions (pH = 3) hydrolyzes proteins into proteoses and peptones. The tests used were not capable of determining whether the enzyme was identical with mammalian pepsin.

Fibrin stained in carmin (Hawk, Oser and Summerson, 1947) or 1.0 per cent Amaranth solution (Swingle, 1928) was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of the water extracts of juices from lumina of the several divisions of tracts of the two fishes was added a drop of congo red indicator, which produced a red color. This indicated a pH value of approximately 5. Sufficient 0.2 *N* HCl was added to cause congo red to turn blue—a pH of approximately 3. A small piece of the colored fibrin was placed in each of the test and control samples. Then the samples were covered with toluene and incubated at room temperature. Digestion was indicated if gradual dissolution of fibrin occurred.

In goldfish, pepsin was found to be absent from the digestive tract and the associated glands, since the fibrin was unaffected even after several weeks of incubation in the extracts.

In largemouth black bass, however, pepsin was present in both the extract from the mucosa of the stomach and the extract of juice from the lumen of the stomach. The action was considered slow, for it took two days for the small piece of fibrin to be completely digested. This slow action possibly could be attributed to reduced secretory activity of the glands, associated with low rate of feeding of the largemouth black bass during the cold weather of February and March.

Trypsin

In this work, trypsin was assumed to be present if digestion of proteins occurred under strongly alkaline conditions (pH = 8.4). The tests used were incapable of determining whether this enzyme was identical with mammalian trypsin.

Fibrin stained in congo red (Hawk, Oser and Summerson, 1947) or 1.0 per cent Ruthenium Red solution (Swingle, 1928) was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of water extract of juices from lumina of the several divisions of the digestive tracts of the two fishes were added 2 milliliters of water and 2 drops of cresol red indicator. Sufficient 0.2 *N* sodium carbonate was added to bring the cresol red to a faint pink, a pH of approximately 8.4. Each test and control sample received a small piece of colored fibrin, was covered with toluene, and was incubated at room temperature. Digestion was indicated by the gradual dissolution of the piece of fibrin.

In goldfish, trypsin was found to be present in extracts from hepato-pancreas, intestinal bulb, anterior intestine, and posterior intestine; and also in extracts of juices from lumina of the intestinal bulb, the anterior intestine, and posterior intestine. This indicated that in the absence of pepsin, trypsin was the only active protein-hydrolyzing enzyme, and was secreted not only in the hepato-pancreas but also in almost the entire length of the digestive tract except the oesophagus and the

rectum. Tissue extracts digested the fibrin within five to seven days. The extract of juice from the lumen of the intestinal bulb digested fibrin within 18 hours. Extracts of juices from the lumen of the anterior intestine and from the lumen of the posterior intestine took 24 to 36 hours and four to seven days, respectively, to complete digestion of similar pieces of fibrin. The rapidity of tryptic action in the extract of juice from lumen of the intestinal bulb may be related to the fact that the secretions from both the hepato-pancreas and the mucosa of the intestinal bulb itself pour into the lumen of this part of the tract.

In largemouth black bass, trypsin was found to be present in extracts from pancreas, liver, and pyloric caeca, and in extracts of juices from lumina of the stomach, duodenum, and small intestine. The extracts of juices from lumina of stomach, duodenum, and small intestine were found to be very active, for the fibrin was completely digested within 18 to 72 hours; the greatest activity was found in the extract of juice from the lumen of the stomach, wherein the fibrin was digested within 18 hours. The period for complete digestion of similar pieces of fibrin in extracts from mucosae of stomach, duodenum, and intestine ranged from five to ten days.

Rennin

In the presence of this enzyme, the casein of milk is hydrolyzed into soluble paracasein. The soluble paracasein combines with the soluble calcium salts of milk and an insoluble curd of paracasein results.

Fresh milk was used as substrate. To 1 milliliter of glycerol extract of each of the several tissues and to 1 milliliter of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of fresh milk. Test and control samples were covered with toluene and incubated at room temperature. Action of the enzyme is indicated by curdling of milk.

In both the goldfish and the largemouth black bass, rennin was found to be absent in the digestive tracts and the associated glands, since there was no separation of curd even after several weeks of incubation of milk in the extracts.

Amylase

In the presence of this enzyme, starch is hydrolyzed into dextrin and ultimately into maltose. Presence or absence of starch is indicated by the iodine test and the presence of reducing sugar (maltose) by the Fluckiger test as outlined by Swingle (1928).

One per cent freshly prepared starch solution was used as substrate for the iodine test. To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of starch solution. Several drops of I_2-KI solution (0.5 per cent I_2 in 1.5 per cent KI solution) were added in each of the test and control samples. The samples were covered with toluene and incubated at room temperature. Digestion was indicated by the solution turning colorless. Loss of color was very often found to be due to absorption of iodine by the tissue suspension and not due to digestion of starch. Upon addition of a few drops of I_2-KI solution the color again developed. In such cases, in-

cubation was continued with frequent addition of drops of I_2-KI solution until no blue color developed, at which stage digestion of starch was complete.

In the Fluckiger test for reducing sugar, a drop of 20 per cent NaOH was mixed with about an equal quantity of powdered copper tartrate upon a microscope slide until the powder was dissolved. A drop of the incubated starch solution was added and then the slide was gently warmed. Presence of maltose was indicated by a red precipitate of copper.

In the goldfish, amylase was found to be present in tissue extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in extracts of juices from lumina of the intestinal bulb, the anterior intestine, and the posterior intestine. This indicated that amylase, like trypsin, was secreted not only in hepato-pancreas but also in a major part of the mucosal lining of the digestive tract. Tissue extract from hepato-pancreas took 24 hours to digest the starch completely. The actions in the rest of the extracts were less rapid and quite variable. It took two to four days for the starch to become completely digested.

In the largemouth black bass, amylase was found to be absent in the digestive tract and associated glands.

Maltase

In the presence of this enzyme, maltose is hydrolyzed into glucose, a reducing sugar. Benedict's solution becomes filled with red, yellow, or green precipitate of cuprous oxide, depending upon the amount of sugar present. If no glucose is present the solution remains perfectly clear. A counter check was run by use of Barfoed's solution (Hawk, Oser and Summerson, 1947), which is also a copper reduction test. Since maltose is a reducing sugar, careful control of the heating times in both Benedict's and Barfoed's test was used to differentiate this sugar from glucose.

One per cent freshly prepared maltose solution in distilled water was used as substrate in both the tests. The distilled water was thoroughly boiled to eliminate carbon dioxide.

To 1 milliliter of glycerol extract of each of the several tissues and to 1 milliliter of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of maltose solution (Benedict's test). The test and control samples were covered with toluene and incubated at room temperature for 24 to 96 hours. After incubation the test and control samples were filtered. The filtrates were tested for glucose. To 5 milliliters of the Benedict's solution in a test tube, exactly 8 drops of the filtrate were added and mixed well. The mixture was placed in boiling water for three minutes and then allowed to cool. In the presence of glucose, a red, yellow, or green precipitate of cuprous oxide was formed.

To 5 milliliters of Barfoed's solution was added 1 milliliter of filtrate prepared as previously indicated (Barfoed's test). After mixing well it was boiled for 30 seconds and then allowed to cool. In the presence of glucose, a red, yellow, or green precipitate of cuprous oxide was formed.

In goldfish, maltase was found to be present in extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in

extracts of juices from lumina of intestinal bulb, anterior intestine, and posterior intestine. This indicated that maltase, like trypsin and amylase, was secreted not only in hepato-pancreas but also in a major part of the mucosal lining of the digestive tract. The digestive action in extract from hepato-pancreas was relatively weak as compared to the action in extracts from the intestinal bulb, anterior intestine, and posterior intestine. This indicated that the role of hepato-pancreas in secretion of maltase was only secondary. The action of extracts from intestinal bulb, anterior intestine, and posterior intestine was variable, being the weakest in extracts from mucosa of the intestinal bulb and from the juice of its lumen.

In largemouth black bass, maltase, like amylase, was found to be absent in the digestive tract and the associated glands.

Lactase

In the presence of this enzyme, lactose is hydrolyzed into glucose and galactose. Benedict's and Barfoed's tests were run to determine presence of glucose. Since lactose is also a reducing sugar, careful control of the heating times was used to differentiate this sugar from glucose.

One per cent freshly prepared lactose solution in distilled water was used as substrate in both the tests.

In both the goldfish and the largemouth black bass, lactase was found to be absent in the digestive tracts and the associated glands.

Invertase

In the presence of this enzyme, sucrose is hydrolyzed into glucose and fructose, both reducing sugars. Benedict's, Barfoed's, and Fluckiger tests were run to determine the presence of the reducing sugars.

One per cent freshly prepared sucrose solution in distilled water was used as substrate in both the Benedict's and Barfoed's tests. The distilled water was boiled to eliminate carbon dioxide. In Fluckiger's test, extracts incubated in 15 per cent sucrose solution for 48 to 96 hours were used. Fructose reduces the copper instantly in the cold. Glucose reduces it upon warming.

In goldfish, invertase was found to be present in extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in extracts of juices from lumina of the intestinal bulb, anterior intestine, and posterior intestine. This indicated that invertase, like amylase and maltase, was secreted not only in hepato-pancreas but also from a major part of the mucosal lining of the digestive tract.

In largemouth black bass, invertase, like amylase, maltase and lactase, was found to be absent in the digestive tract and the associated glands, since the extracts did not respond to any of the several tests run.

Lipase

In the presence of this enzyme, fats are hydrolyzed into fatty acids and glycerol.

Emulsified olive oil containing brom thymol blue indicator and whose pH had been adjusted to approximately 7.2 was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and 2 milliliters of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 2 milliliters of emulsified olive oil after the pH of the extracts had been adjusted to 7.2. The test and control samples were covered with toluene and incubated at room temperature. Digestion was indicated by a change of color of the emulsion from blue to greenish yellow or yellow caused by free fatty acids.

In goldfish, lipase was found to be present in extract from hepato-pancreas, and in extracts of juices from lumina of intestinal bulb and anterior intestine.

In largemouth black bass, lipase was found to be present in extracts of juices from the lumen of the duodenum and small intestine.

General Conclusions and Discussion

These investigations revealed that a correlation existed between the structtures of the alimentary canal and the occurrence and distribution of digestive enzymes. In goldfish, where the hepato-pancreas is the only accessory secretory gland associated with the digestive tract, lipase was evidently of little importance and was found in extracts from the hepato-pancreas alone. Trypsin was the only protease found, and it occurred in extracts from hepato-pancreas, intestinal bulb,

Table I

The digestive enzymes in goldfish and largemouth black bass

Species	Secretory organ	Pepsin	Trypsin	Rennin	Amylase	Maltase	Lactase	Invertase	Lipase
Goldfish	Hepato-pancreas	−	+	−	+	+	−	+	+
	Oesophagus	−	−	−	+	+	−	+	−
	Intestinal bulb	−	+	−	+	+	−	+	−
	Anterior intestine	−	+	−	+	+	−	+	−
	Posterior intestine	−	+	−	+	+	−	+	−
Largemouth black bass	Liver	−	+	−	−	−	−	−	+
	Pancreas	−	+	−	−	−	−	−	−
	Pyloric caeca	−	+	−	−	−	−	−	+
	Oesophagus	−	−	−	−	−	−	−	−
	Stomach	+	−	−	−	−	−	−	−
	Duodenum	−	−	−	−	−	−	−	−
	Small intestine	−	−	−	−	−	−	−	−

Explanation: The + indicates a positive test for the enzyme. The − indicates a negative reaction for the enzyme.

anterior intestine, and posterior intestine. The carbohydrases, amylase, maltase and invertase were found in extracts not only from the hepato-pancreas, but also in tissue extracts from a major part of the length of the digestive tract, including the oesophagus (Table I); they were active in the lumen throughout almost the entire length of the tract (Table II).

The digestive activity of the oesophagus in fishes has been reported by only a few investigators (Sullivan, 1908; Kingsley, 1917). The presence of the three carbohydrases in extracts from oesophagus of goldfish is in conformity with earlier findings (Sarbahi, 1940) of highly complex racemose glands in the mucosa of the

oesophagus of the rohu (*Labeo rohita*), which belongs to the same family (Cyprinidae) as goldfish.

Analyses of extracts of the hepato-pancreas and oesophagus indicated that the former secretes enzymes acting on carbohydrates, proteins, and fats, while the latter secretes enzymes acting on carbohydrates. The great digestive power of the secretion from the hepato-pancreas and the assumption of digestive activity by the oseophagus is closely associated with the absence of a compact pancreas and a true stomach. In *Labeo rohita* with a similar type of digestive apparatus, it was found (Sarbahi, 1940) that typical gastric glands also were absent.

The wide distribution of carbohydrases as compared to the distribution of proteinases and lipase indicated that goldfish could utilize foods that included a

TABLE II

Site of action of enzymes secreted in goldfish and largemouth black bass

Species	Site of action	Pepsin	Trypsin	Rennin	Amylase	Maltase	Lactase	Invertase	Lipase
Goldfish	Intestinal bulb	−	+	−	+	+	−	+	+
	Anterior intestine	−	+	−	+	+	−	+	+
	Posterior intestine	−	+	−	+	+	−	+	−
Largemouth black bass	Stomach	+	+	−	−	−	−	−	−
	Duodenum	−	+	−	−	−	−	−	+
	Small intestine	−	+	−	−	−	−	−	+

Explanation: The + indicates a positive test for the enzyme. The − indicates a negative reaction for the enzyme.

large percentage of carbohydrates, a fair amount of proteins, and a small amount of fats. The food of goldfish is chiefly plant materials that are rich in carbohydrates and contain fair amounts of proteins.

Foods of plant origin, being low in concentrates as compared to foods of animal origin, have to be consumed in bulk to meet the body requirements. Consumption in bulk implies longer retention in the tract for complete digestion. This involves the presence of a comparatively long digestive tract, such as is found in goldfish.

In largemouth black bass, proteinases are evidently the chief enzymes. Pepsin was found in extracts from the stomach and trypsin in extracts from liver, pancreas, and pyloric caeca. Lipase was found in extracts from liver and pyloric caeca. None of the carbohydrases was found in extracts either from glands associated with the digestive tract or from the tract itself.

The oesophagus of largemouth black bass, unlike the oesophagus of goldfish, had no digestive activity. This is closely associated with the presence of a compact pancreas and a true stomach.

The largemouth bass has a shorter digestive tract than that of the goldfish but has additional sources of enzymes to expedite digestion.

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